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I. THE AUTOLYSIS OF THE MUSCLE OF THE COD FISH.

By ERNEST HAROLD CALLOW.

A REPORT TO THE FOOD INVESTIGATION BOARD, DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH, FROM THE LOW TEMPERATURE RESEARCH STATION, CAMBRIDGE

(*Received October 9th, 1924.*)

Fearon and Foster [1922] showed that ox muscle, after being frozen rapidly in brine at − 8° and then thawed, autolysed at about the same rate (in the presence of toluene) as normal muscle. On the other hand, muscle which had been frozen slowly in air at − 8° and then thawed showed a greater amount of autolytic change and a more rapid rate of autolysis. The present investigation is an extension of Fearon and Foster's work to the muscle of cod. No reference to the autolysis of cod muscle could be found in the literature. The material proved to be very difficult to manipulate and it was only after much preliminary work that satisfactory methods were worked out.

Methods.

The following procedure was finally adopted. The muscle, with connective tissue, was minced by passing twice through a very fine mincer, and then mixed thoroughly by hand; 400 g. were quickly weighed out for the digest. At the same time about 15 g. were transferred to a stoppered weighing bottle and used for determinations of total nitrogen; in each case six estimations of total nitrogen were carried out, the average being adopted. The 400 g. of mince were transferred in small quantities to a mortar and triturated with toluene until each portion was saturated, 150 cc. of toluene being used in all. The mixture was then quantitatively transferred to a sterile 2-litre flask, the total volume of suspension being made up to 2 litres with distilled water. The flask was shaken at intervals for two hours. After this, a sample was withdrawn and the flask placed in the incubator or cold room. Every 12 hours the flask was shaken vigorously, and every 24 hours two samples were withdrawn with a blunt-ended pipette delivering 24·7 cc. Each sample was placed in a stoppered 100 cc. measuring flask containing 50 cc. of a 4 % solution of trichloroacetic acid. The contents of the flask were made up to 100 cc. by addition of distilled water. The mixture was left for 24 hours and then filtered. Estimations of the nitrogen content of the filtrate (so called "soluble nitrogen") were made by Cole's alcohol distillation method [Cole, 1920]. Nitrogen determinations of the reagents were carried out and due allowance made.

In order to compare fresh and frozen muscle, a fish was bisected longitudinally. Muscle was taken from one half and minced for autolysis; this served for a control. The other half was frozen, stored, thawed and then minced for autolysis. For the processes of freezing and storing, halves of two different fish were placed together in an oiled-silk bag, so that the thickness would be the same as that of a whole fish. Thus the fish was affected neither by penetration of salt during brine freezing, nor by desiccation during air freezing and storage.

EXPERIMENTAL RESULTS.

Exp. 1. *The Autolysis of Fresh Cod at* + 37°. In order to test the accuracy of the method, preliminary autolyses were carried out. The following experiment shows that the experimental error involved is quite small.

Fresh cod was minced in the usual way, and duplicate autolyses were carried out. Fig. 1 shows that such duplicates gave practically identical results, thus illustrating the accuracy of the method.

Exp. 2. *The Autolysis of Fresh Cod at* + 1°. Fresh cod was minced and put up in four similar flasks, two of which were incubated at + 37° and sampled daily. The other two flasks were kept in a room at + 1° and samples were removed at intervals of several weeks. The experiment commenced on October 5th, 1923.

Table I.

	Soluble N	
	Flask 1	Flask 2
Date examined	%	%
October 9, 1923	10·9	10·9
January 7, 1924	10·8	10·9
May 23, ,,	11·7	11·7
October 5, ,,	12·4	12·2

The results of the experiments at + 37° are shown in Fig. 1 whilst Table I shows the results at + 1°. It will be seen that the rate of autolysis at + 1° is very much reduced. From these data it was calculated that the rate of autolysis at + 37° was 223 times more rapid than at + 1°. (This gives a temperature coefficient of 4·5 for each 10°.)

Exp. 3. *The Effect of Brine-freezing at* − 19° *on the Rate of Autolysis.*

Halves of two different fishes were placed together in an oiled silk bag and frozen in brine at − 19° for $2\frac{1}{2}$ hours, and then stored at − 11° for 10 days. They were thawed by hanging in air at + 1° for 96 hours, then minced and autolysed at + 37° in the usual way. The other halves of the two fishes were minced without having been frozen, and incubated to give a measure of normal autolysis.

Fig. 2 shows that the brine-frozen fish gave results practically identical with those of the unfrozen control. This agrees with the results of Fearon and Foster [1922] who found that brine-freezing does not cause any appreciable change in the tissues of ox muscle.

Exp. 4. *The Effect of Air-freezing at* – 11° *on the Rate of Autolysis.* Two cod were bisected longitudinally. Half of each fish was immediately minced for a control experiment, suspensions being made in the usual way and incubated at + 37°. The other halves were placed together in an oiled-silk bag and slowly frozen by hanging in a cold chamber at – 11° for 10 days. Portions of muscle were then cut from each half-fish and placed in two separate covered beakers. The beakers were placed in a room at + 1° for 66 hours to ensure slow thawing. During this process liquid ("drip") separated out. The muscle was then minced and the mince and "drip" thoroughly mixed together. Suspensions were made up and autolyses were carried out at + 37° in the usual way.

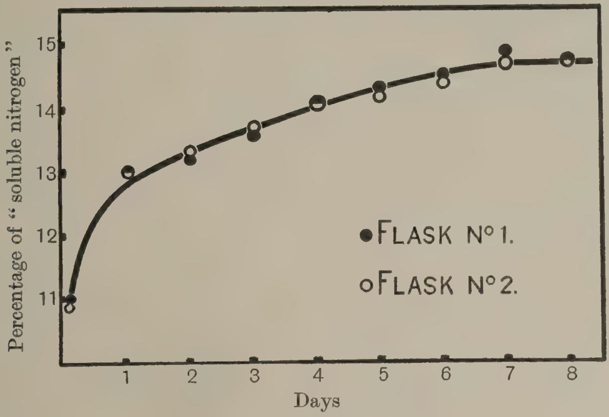

Fig. 1. Duplicate autolyses

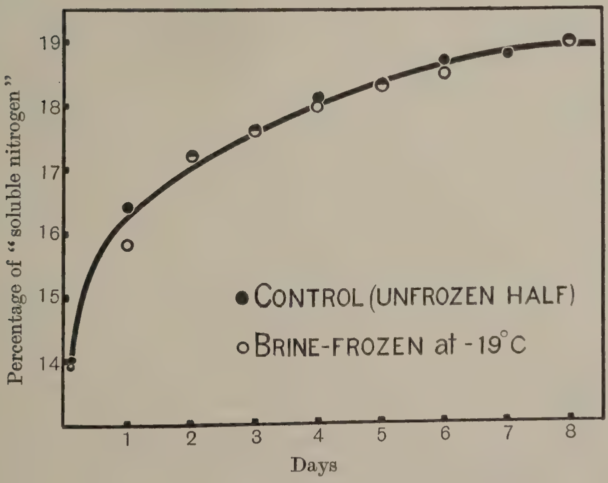

Fig. 2. The autolysis of brine-frozen cod muscle

The results of a typical experiment (see Fig. 3) show that in the case of air-frozen cod there is slightly more autolytic change than occurs in the unfrozen control. This result is also in agreement with Fearon and Foster's [1922] conclusions on the air-freezing of ox muscle.

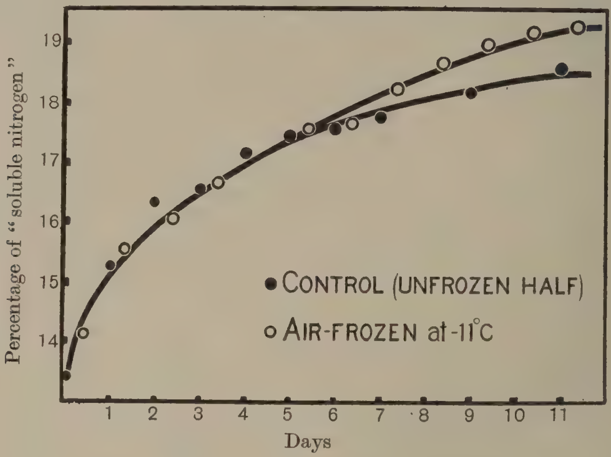

Fig. 3. The autolysis of air-frozen cod muscle

Exp. 5. *The Autolysis of "Drip."* "Drip," that is the fluid exuded by slowly frozen tissues on thawing, was collected from a cod frozen in air at – 19°. 100 cc. of this fluid were mixed with 37·5 cc. of toluene and the volume made up to 500 cc. with distilled water. The mixture was incubated at + 37° and samples were withdrawn daily and analysed. It was found that "drip" contained a higher initial percentage of soluble N (27·9 %) than cod muscle, and that the *increase* of soluble N (to 36·4 %) during the course of autolysis was also greater than in the case of muscle.

DISCUSSION.

Previous work [Stiles, 1922; Richardson and Scherubel, 1908; Kallert, 1923] on the freezing and thawing of animal tissues has shown that the minimum of alteration is obtained when the tissues are frozen rapidly and thawed slowly. The reason for this is that during freezing ice crystals separate out—the slower the freezing the larger the ice crystals formed and *vice versa*.

Obviously the ideal method is to freeze so rapidly that the size of the ice crystals is a minimum. Brine freezing at low temperatures appears to give a rate rapid enough for most practical purposes.

It has been observed that, on thawing frozen tissues, the water formed from the ice is slowly reabsorbed by the tissues [Kallert, 1923; Richardson and Scherubel, 1908.]

Even after complete thawing has been accomplished, this process of absorption still goes on. In the present investigation this fact was borne in mind, the muscle tissues being given ample opportunity to reach equilibrium before the autolyses were carried out.

The results of this investigation show that air-frozen cod suffered a much greater destruction of its tissues than brine-frozen cod. Cod muscle frozen rapidly in brine at – 19° gave no "drip" and presented a perfectly normal appearance on thawing, whereas cod frozen slowly in air at – 19° and – 11° always "dripped" on thawing and felt noticeably flabbier. The volume of "drip" which could be collected in such cases was of the order of 10 % of the weight of the fish. Also, rapidly frozen cod muscle autolysed at the same rate as the unfrozen control whilst with slowly frozen cod muscle there was a slight but appreciable increase in the amount and rate of autolysis. Thus for cod muscle as for other animal tissues the best method of preservation is to freeze rapidly.

Experiments carried out at + 1° showed only slight autolysis even after 12 months. This is of importance as it indicates that no appreciable changes are likely to occur in the proteins of *frozen* cod stored for prolonged periods at low temperatures such as – 11°.

Note on a Protein of Cod Muscle.

When "drip" was incubated at + 37° with toluene, a coagulum was observed after 24 hours. It consisted chiefly of protein. The following experiments with cod muscle offer further evidence for the existence of a protein which coagulates at + 37°.

1. Air-frozen cod muscle whilst still in the frozen condition was minced, suspended in a 5 % solution of $MgSO_4 7H_2O$ and left over night. The suspension was then filtered and the filtrate gently heated to + 37·5° when a faint opalescence was observed. Another experiment on the same filtrate gave an opalescence at + 38°.

2. The following experiment proved that the presence of $MgSO_4 7H_2O$ had no appreciable effect on these results. Shavings of air-frozen cod were ground up in a mortar with sand and distilled water. The filtrate from this extraction also contained a protein which commenced to coagulate at + 36°. This experiment was repeated with fresh cod with the same result.

SUMMARY.

1. At + 37° autolysis of cod muscle was practically complete after seven to eight days but at + 1° autolysis proceeded very slowly and even after 12 months the changes were quite slight.

2. Cod frozen rapidly (in brine at – 19°) presented a normal appearance on thawing, and on autolysis at + 37° gave results identical with those of the unfrozen control.

3. On thawing cod which had been frozen slowly (in air at – 11°), a fluid ("drip") separated out. Autolysis of the thawed tissue with the appropriate amount of "drip" proceeded at a slightly greater rate than that of the unfrozen control.

4. "Drip" was found to contain a higher initial percentage of soluble N and to autolyse to a greater extent than normal cod muscle.

5. Cod muscle was found to contain a protein which coagulates at + 37°.

My thanks are due to Professor F. G. Hopkins, F.R.S., for the interest he has shown in the investigation.

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II. THE PREPARATION AND PROPERTIES OF PURIFIED OLEIC ACID AND SOME OF ITS SALTS.

By ARTHUR LAPWORTH, LEONORE KLETZ PEARSON
and (in part) EDWARD NEVILLE MOTTRAM.

From the University, Manchester.

(*Received November 12th, 1924.*)

Notwithstanding the large amount of work which has been done on the chemistry of oleic acid the published methods for preparing it leave much to be desired. The descriptions given of the compound itself inspire so little confidence that even the most recent edition of the Handbook of Lewkowitsch [1921] quotes melting points for oleic acid varying from 6·5° to 16°, although Kirschner's conclusion [1912] that the acid is dimorphous, is accepted. Edmed [1898] by recrystallisation of Kahlbaum's oleic acid from alcohol obtained a sample of the acid which was stated to melt at 13–15°. Partington [1911] by crystallising another specimen obtained from Kahlbaum, and probably one of inferior grade, could not obtain a product melting higher than 6·5°; Kirschner [1912] by following much the same procedure obtained samples of the acid which showed dimorphism and therefore two different melting points. The present authors originally hoped to devise an improved method for the estimation of oleic acid in mixtures such as are obtained by hydrolysis of natural fats and oils, but soon found that it would be necessary to study the question of preparing and characterising pure oleic acid almost *ab initio*.

During the course of their studies, now extending over some years, the authors have been forced to retrace their steps many times, but they believe that they can now with advantage place some of their results on record and indicate with considerable confidence the probable margins of experimental error. Having tried nearly all the published devices for purifying oleic acid and numerous combinations and permutations of these and others, they have worked out a process by which oleic acid containing not more than 1 % of impurity can, with practice, be obtained with considerable certainty.

Separation of salts of higher fatty acids by recrystallisation.

The authors have latterly confined their experiments to the acids obtained by hydrolysis of olive oil, in which it is known that palmitic acid is the main saturated constituent. It is not intended to discuss this point in the present paper, but it may be stated that the authors have never detected the presence of any saturated acid higher than palmitic acid in samples of oleic acid made from olive oil by the process recommended, so that stearic acid, for example, if present in the crude acid, was mainly eliminated as lead salt in the first stages of purification. In addition to oleic acid, palmitic acid and any acids which are easily eliminated owing to the sparing solubility of their lead salts, there are present linolic acid and perhaps others still more highly unsaturated. [Compare Hehner and Mitchell, 1896.]

Various metallic salts of oleic acid, linolic acid and palmitic acid with barium, lead, lithium and sodium have been examined by the present authors especially with regard to their suitability for the purpose of separating the acids from one another. It is unnecessary to go into the large mass of experimental detail and it will be sufficient to say that when a mixture of palmitic acid and oleic acid was converted into barium, lead, lithium or sodium salts and these were fractionated by crystallisation, the palmitate was invariably found in larger proportion in the solid than in the dissolved portions. This behaviour is quite consistent with solubility curves for solid solutions of certain types and renders it highly improbable that any recrystallisation process applied to these salts can yield pure oleates[1].

It has been suggested by C. W. Moore [1919] that the use of salts of bivalent metals for separating oleic acid from other acids is to be avoided owing to the possibility of double salts, such for example as barium oleo-linolate, being formed. The objection would doubtless hold good if barium oleo-linolate, for example, were less readily soluble than barium oleate, but as the mixed salts invariably, in the experience of the present authors, exhibit a much higher degree of solubility than the pure oleates, the difficulty is not a serious one from the theoretical point of view, and in actual practice the purification of barium oleate from barium linolate by recrystallisation is comparatively easy.

Properties of mixed lead salts of oleic and other fatty acids.

The separation of palmitic acid and other saturated fatty acids from oleic acid is as a rule effected by making use of the greater solubility of lead oleate as compared with palmitate, in ether, benzene or petroleum, usually the first. It is widely known that this method is imperfect but it is perhaps not generally realised that it is very rough indeed. The solubility of lead palmitate in ether, benzene and other solvents is not diminished as might be anticipated, but is in fact greatly increased by the presence of lead oleate, linolate, etc. Unless, therefore, care is exercised to remove, with the lead palmitate, a large proportion of the oleate itself, the oleic acid prepared by the lead salt process may contain 5 % or even more of palmitic acid. On freezing the solution, however, the lead oleate which then separates out carries down with it a large proportion of the remaining lead palmitate and finally an oleic acid can be isolated from the mother liquor which does not contain more than about 2 % of palmitic acid, which is the smallest proportion of palmitic acid which it has yet been found practicable to obtain in any sample of oleic acid purified by a process involving crystallisation only.

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[1] The forms of the freezing point "curves" of mixtures of oleic and palmitic acid (compare Fig. 1, p. 15) also indicate that any complete purification of the former by a recrystallisation process applied to a mixture of the free acids would not be practicable.

It must be emphasised too, that the solubility of lead oleate, for example, in ether, varies considerably not only with the quantity of other salts present but apparently also with the quantity of alcohol and of water present and also with time. A clear solution of freshly precipitated, crude lead oleate in ether, if left for some days or weeks in a closely stoppered vessel, gradually deposits much of the salt in solid form and the deposit is then comparatively very sparingly soluble in ether. The cause of this phenomenon is obscure.

If a fresh ethereal solution of the mixed lead salts from olive oil be filtered at ordinary temperatures and then cooled, amorphous lead oleate mixed with palmitate tends to be deposited above − 10°, but below that temperature the deposit tends to become crystalline and on warming to ordinary temperatures does not always wholly redissolve. The property which lead oleate has of becoming crystalline below − 10° may be taken advantage of, if desired, to remove the bulk of the more highly unsaturated acids, as a preliminary in preparing oleic acid from olive oil, using ether as a solvent. In this case the ethereal solution remaining after the bulk of the palmitate has been separated by filtration at ordinary temperatures is slowly cooled to − 15° with constant agitation, and as soon as the deposit has become granular[1] the whole may be filtered through a Buchner funnel with the aid of a pump; the physical condition of the crystalline deposit is so well fitted for rapid filtration that when using several hundred grams of ether the solid may be secured without difficulty or serious loss of solvent even if no special precautions are taken to cool the funnel beforehand. In this way the bulk of the lead salts of the more highly unsaturated acids may be removed and subsequent processes of purification to some extent facilitated.

Barium oleate.

Farnsteiner [1901] first drew attention to the fact that barium oleate, although nearly insoluble in hot benzene containing absolute ethyl alcohol, dissolves if a very little water is present, and comes out on cooling in lustrous needles or long laminae. He claimed that a method for the quantitative separation of oleic acid from more highly unsaturated fatty acids could be based on this, as the barium salts of these remained dissolved; this conclusion however was disputed by Lewkowitsch [1903] who stated that, in presence of these acids, barium oleate is soluble in the medium. The experience of the present authors agrees with that of Lewkowitsch, and in another paper data will be furnished which fully substantiate his statement. The barium salt of oleic acid may be made use of, however, for the isolation of highly purified oleic acid from a mixture which contains a large proportion of that acid—such as that from olive oil. The authors find, however, that several great advantages are to be gained by substituting amyl alcohol for ethyl alcohol. When ethyl alcohol is used there is danger of the water, present or added, causing the alcohol to separate in diluted form as a distinct phase; with amyl alcohol on the other hand, if there is excess of water, it is the excess of water which remains as the separate phase and has no ill effect. Toluene was used in the later experiments rather than benzene as it is less volatile; the mixture of toluene and amyl alcohol is readily recovered with but little change in composition, by distillation in a current of steam, and used again as solvent. A moist mixture of equal volumes of toluene and amyl alcohol is recommended for rapid purification of barium oleate, but toluene with 10 % of amyl alcohol may be used.

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[1] Repeated warming and cooling is sometimes necessary to obtain a granular material.

Dry barium oleate is practically insoluble in benzene or toluene even on boiling. If a little of this salt is heated with either of these liquids in a test tube and a drop of water is added the powdery particles of the salt swell and soon the whole forms an opaque jelly and remains as a jelly when cool. On subsequent addition of one drop of amyl or ethyl alcohol and again heating, a thin, slightly opalescent solution is at once formed, and this on cooling deposits the salt in shimmering flat needles. The salt is soluble in a hot mixture of nearly dry amyl alcohol and benzene (or toluene) but on cooling comes down as a powder, which has no crystalline appearance unless a little water is first added.

The solubility of pure barium oleate in a moist mixture of equal volumes of amyl alcohol and benzene was found to be 0·219–0·224 g. per 100 cc. at 12°, determined about 12 hours after cooling a warm saturated solution to that temperature; it may be considerably less if the time of standing be prolonged. This, although small, is nearly fifteen times as large as that found by Farnsteiner for his solvent mixture of benzene and moist ethyl alcohol at 11° [1901]. This greater solubility extends to the other salts present in crude barium oleate, and the fact presents a definite practical advantage when the separation of barium linolate and similar salts from barium oleate is concerned, for these impurities in the oleate are in consequence more rapidly removed on recrystallisation[1]. For rapid elimination of the salts of the more highly unsaturated acids the filtrations are best carried out within an hour or two of cooling to 12°, but it is even better to cool no lower than to 20° and filter at once.

The process which the authors now use for preparing the most highly purified oleic acid is as follows.

ISOLATION OF OLEIC ACID FROM OLIVE OIL.

(With Edward Neville Mottram.)

Olive oil (400 g.) is heated on the steam-bath under a reflux condenser for $3\frac{1}{2}$–4 hours with spirit (800 g.) and water (200 cc.) containing excess of sodium hydroxide. The whole is then cooled and its alkalinity nearly but not quite destroyed by addition of acetic acid, using phenolphthalein as indicator. A strong aqueous solution of lead acetate is slowly added while the whole is agitated, when a precipitate is formed and adheres to the walls of the containing vessel; the addition of lead acetate is discontinued when no further precipitation ensues. The supernatant liquid is poured off and worked up to recover alcohol, the lead precipitate is fairly well drained and then dissolved in toluene (1 litre) with the aid of a steam-bath. The toluene solution is now heated for some time with solid dehydrated lead acetate (50 g.)[1]; this dissolves, but on cooling to ordinary temperature separates once more together with some 20 % of the whole quantity of the other lead salts originally present (crude lead "palmitate")[2].

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[1] When Farnsteiner's mixed solvent is used for the latter purpose, it is almost impossible wholly to remove the more soluble barium salts by a limited number of crystallisations, unless the filtrations are carried out at temperatures not lower than 38–40°.

After cooling the solution to ordinary temperatures and filtering from a residue of about 75–80 g. of insoluble lead salts, the clear toluene solution is shaken with excess of cold, dilute hydrochloric acid until no more lead chloride is formed on removing a portion of the upper solution and agitating with dilute hydrochloric acid. It is not difficult to separate the toluene solution of fatty acids from the aqueous layer in spite of the presence of finely divided lead chloride, and filtration through several layers of filter paper in a large Buchner funnel may be resorted to if necessary. The clear solution is subsequently agitated with water until quite free from hydrochloric acid, separated, and the solvent removed by distillation, finally under diminished pressure (15–20 mm.) on the water-bath.

The acids are now subjected to fractional distillation under a pressure not exceeding 2–3 mm.[3] when the middle fractions are used to prepare the purest oleic acid, care being taken to reject a larger quantity of the earlier fractions than of the later ones, as the main impurity, which the operation is used to eliminate, is the relatively volatile palmitic acid[4]. The earlier portions of the distillate set to a nearly solid mass (mixed crystals of oleic and palmitic acid) on cooling and it is of interest to observe that the authors did not discover any solid saturated fatty acids in the least volatile portions of the material although, when the lead salt separation as a preliminary was omitted, such acids separated out from the residue in the flask[1].

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[1] The lead acetate is used to effect the complete conversion of any remaining sodium salts into lead salts and it also has the effect of clearing the toluene solution and rendering the crude lead "palmitate" easy to filter off. The excess is easily recovered by washing this crude lead "palmitate" with water.

[2] By means of a freezing mixture more lead salt, rich in palmitate, separates out, and the more of this which can be removed, the simpler does the subsequent removal of palmitic acid by fractional distillation become. The removal of 50 % of all the dissolved salts has yielded the best results, but in this case ether is much to be preferred to toluene as solvent. Some lead oleate, contaminated with much palmitate, may be obtained by extracting this crude lead "palmitate" with toluene, benzene or ether, and may be reconverted into free acid, and added to the next batch of oleic acid, before subjecting the latter to the lead salt separation process.

[3] The authors used a very simple fractionating column 15 inches in length and about 1¼ inches in diameter made by the "Scientific Glass-blowing Company, Manchester," which has proved very suitable for fractional distillations under reduced pressure, and was fused to the flask. The required reduction in pressure was attained by means of a mercury vapour pump of the Völlmer type in series with an ordinary Sprengel-Bunsen water pump.

[4] The authors collected their distillates in the first fractionation in two main parts, in order of passing over, representing respectively about (I), one-third and (II) one-half of the total quantity of acid, a dark orange residue (III) representing rather more than one-sixth of the

The fractions selected are now treated in the following manner. A mixture of equal volumes of toluene and amyl alcohol, weighing roughly ten times as much as the oleic acid to be worked up, is heated to water-bath temperature with a pinch of phenolphthalein and rather more powdered crystallised barium hydroxide than is required to neutralise the oleic acid. The oleic acid is added to the hot suspension, and the whole shaken. Barium oleate is formed very quickly and, when the whole appears permanently suffused with the colour of alkaline phenolphthalein, it is allowed to settle. The nearly clear supernatant liquid is poured off while still hot and allowed to cool, when the barium oleate separates, usually in beautifully crystalline form; it is filtered off, washed with toluene and drained.

For the purification of the barium oleate, the drained salt is recrystallised from about two-and-a-half times its weight of a hot mixture of amyl alcohol and toluene in equal volumes, with addition on each occasion of a few drops of water, the process being repeated until 100 g. of the filtrate (filtered after remaining in the cold for about twelve hours at about 12°) yields not more than 0·21 g. of residue on evaporation. From two to five recrystallisations are usually necessary and the salt should be washed with toluene on each occasion[2]. The salt requires now only to be redissolved, separated from insoluble impurities by filtration, and finally freed from adherent solvent by exposure to air or in a vacuum (if desired, after washing with ether). About 90–100 g. of barium oleate containing 2 or 3 % of barium palmitate is thus obtained. Before conversion into free acid the salt must be freed entirely from all odour of amyl alcohol and then suspended in cold water containing

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original quantity of acid remaining in the distilling flask. Fraction (II) was refractionated and the corresponding fraction of this worked up for the purest oleic acid. The fractions not used for conversion into barium salt will yield more good oleic acid by repeating the whole process *ab initio*.

On a large scale, with the incomparably more efficient fractionating columns available in technical work, middle fractions could no doubt be obtained in one operation even purer than those obtained by the authors in two operations and representing a much larger proportion of the crude acid distilled.

[1] It is very doubtful whether these contained stearic acid, which has been stated by some workers not to be present in oleic acid from olive oil. It may be that they are acids higher than stearic acid, in which case it may prove satisfactory on the large scale to omit the lead salt separation process and trust to the fractionating process alone to effect the removal of all saturated acids; in view of the conflicting statements before mentioned, however, and the comparison of the results actually obtained on the small scale with and without the lead salt separation process, the present authors are not yet prepared to recommend the omission.

[2] The purification of the barium oleate from the more soluble barium salts is effected in a smaller number of recrystallisations if the temperature is not allowed to fall too low and if the filtrations are carried out as soon as possible after the selected temperature has been reached. A temperature of 20° is suitable, but if too high, clogging of the filter may occur.

a slight excess of 10 % hydrochloric acid. In the course of some hours, with frequent shaking, much salt is decomposed and if the whole be filtered through a layer of filter paper with the aid of a pump any unchanged salt may be recovered. The oily layer in the filtrate is separated and shaken repeatedly with dilute hydrochloric acid and then with water until free from barium. This mode of working, if somewhat tedious, prevents any contamination of the oleic acid with solvents or contained impurities.

The washed oleic acid is collected and after being freed from water as far as possible by mechanical means is transferred to a distilling flask, dried in a vacuum with the aid of very gentle warmth and fractionated under a pressure of not more than 2–3 mm. The great bulk distils almost constantly, usually between 170° and 175° when using a Völlmer glass mercury vapour-jet pump in series with a Sprengel water pump. About 53 g. are obtained from 100 g. of fractionated oleic acid (crude).

The bulk of the distillate should set readily, on cooling below 11°, to an ice-like mass, the thermometer rising to and remaining practically constant at not lower than 12° and sometimes as high as 12·8°[1].

Oleic acid obtained by the authors when using the above process of purification contains about 2 % of palmitic acid, which reduces its setting point by only about 0·5°. It also contains, however, small quantities of other impurities of unknown character, which are probably products of decomposition of oleic acid by heat and which further affect its setting point by several tenths of a degree; these may for the most part be removed however by draining on porous earthenware, in a vacuum, at 8–10°, while the palmitic acid cannot be so removed.

Properties of oleic acid.

Oleic acid, as Kirschner [1912] has shown, is dimorphous. This dimorphism is exhibited only by moderately pure oleic acid, and is often manifested during solidification, by the setting in of a slow rise in temperature at some stage after the rapid rise after supercooling to the steady setting point of the low melting modification. A constant higher setting point never seems to be reached in practice, however, and this is doubtless because change from the first to the second solid modification is very slow.

In other cases the oleic acid sets to an icy mass and only after some hours displays the phenomenon shown so well in the photograph given in Kirschner's paper, namely, the development of white, opaque sub-spherical aggregates of minute needles, into which the whole is ultimately transformed.

The highest melting point observed for the more stable form was 17·45°, the sample being one which, with an original setting point of 12·04°, had been left for some months during the winter. Another sample, with an original setting point of 12·8°, was found to melt at 17° after remaining some days in the solid state.

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[1] Several per cent. of palmitic acid do not greatly affect the melting point of oleic acid, but a high melting point is a fairly good guarantee that the more highly unsaturated acids and other impurities are absent.

The best samples of oleic acid made by the present authors, if slowly cooled while in the fluid condition, began to deposit crystals when the temperature had fallen to about some point between 12·3° and 12·8° and the temperature then rose quickly to a maximum, usually remaining then almost constant until the whole was nearly solid[1]. It was very seldom that this supercooling was greater than 0·65–0·7°, but Kirschner was able to keep his oleic acid at 8° for some hours and could find no temperature of spontaneous crystallisation. Kirschner, however, prepared his oleic acid by the repeated partial solidification and centrifugalisation of Kahlbaum's purest preparation of oleic acid, some samples of which he found to deposit a solid which had the characters of elaidic acid; his acid was therefore apparently saturated with elaidic acid and may have contained other fatty acids capable of forming isomorphous mixtures with oleic acid without greatly affecting its melting point.

SOME FURTHER OBSERVATIONS ON THE PROPERTIES OF PURIFIED OLEIC ACID.

Mr Edwin Richardson has observed that when freshly distilled oleic acid contained in a glass vessel is exposed to diffused daylight for about twelve hours a small but definite depression of the setting point is often observed, amounting to about 0·07–0·1°. After this initial period further change is very slow and even when the acid is exposed to air, or when dry air is bubbled through it at ordinary temperatures for five hours, the setting point is lowered by only one or two hundredths of a degree. On the other hand, the setting point of a sample which had been left in a closely-stoppered glass bottle for about a year had fallen by about 1°.

Saturation of dry, purified oleic acid with water leads to a depression in the setting point of about 0·35° (mean of 4 concordant observations). Water is less soluble in oleic acid at 12° than at 25°, so that on cooling oleic acid saturated with water, considerable cloudiness is observed.

First setting points of mixtures of oleic and palmitic acids.

A weighed sample, usually amounting to 5–10 g. of each of numerous specimens of drained and undrained oleic acid prepared by processes not quite so efficient as the one above described, was placed in a thin glass tube which was surrounded by another which provided an air-space separating the former from the cooling bath of water. The bulb of the thermometer[2] was immersed in the acid, which was stirred with a platinum spiral. The rate of cooling in any series of observations was kept as nearly as possible constant and the first maximum temperature reached after solidification set in was recorded as the "first setting point," the observation being repeated until consistent results were obtained with a steady rate of cooling. Small weighed pellets of palmitic acid were added, dissolved by warming and stirring and new observations made.

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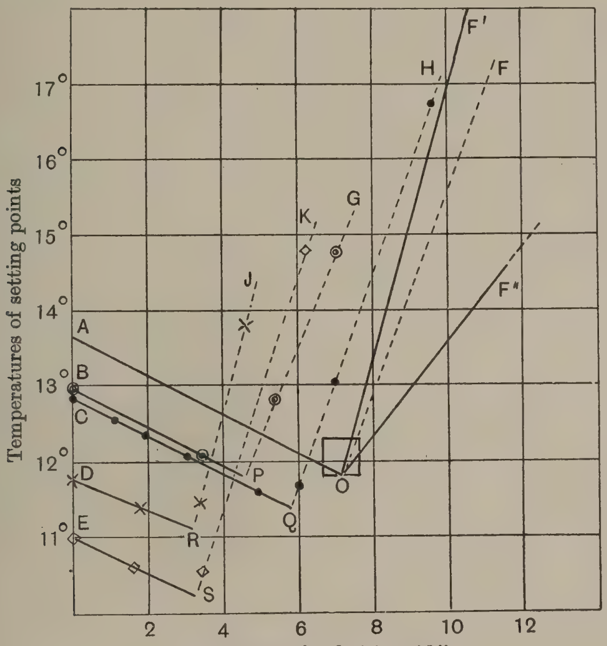
[1] This temperature is what hereafter is termed the "first setting point." When any marked change in temperature from the first setting point was observed, during the solidification of oleic acid containing less than 6 or 7 % of palmitic acid, it was a rise, due to the appearance of the second and higher melting modification.

[2] The temperatures given in this section were taken with a standardised thermometer capable of being read to 0·01° and are given to the nearest 0·05°.



"Percentage of palmitic acid"

Fig. 1.

The general character of the results obtained will readily be understood by reference to Fig. 1, which exhibits actual observations made with four different samples of fairly high grade oleic acid. The *percentage of palmitic acid* was in each case the weight of palmitic acid added divided by the total weight of material present. One of these, giving the points on *BPG*, was selected because it was practically free from secondary impurities; another, *CQH*, because it was the last sample examined and a specially large number of observations was made with it in order finally to decide whether a real break occurred. The other two, namely *DRJ* and *ESK*, illustrate the behaviour of somewhat less pure specimens. The descending branches (reading from left to right) of the setting point "curves" had a slope practically independent of the amount of impurity in the samples examined. The slope of descent was invariably nearly 0·25° for each additional 1 % of palmitic acid. The steeper branches of "curves" actually traced in practice are indicated by interrupted lines, as the slopes of these varied somewhat with the speed of cooling adopted and consequently were more or less fortuitous, but served to determine the approximate position of the "breaks."

The temperature at which the break occurs with each sample would of course always be the same if only oleic acid and palmitic acid were present. The fact that this break-temperature usually appears below 11·8° is due to very small quantities of oily secondary impurities; these are probably of low molecular weight and therefore very effective in depressing the melting point, but apparently with little influence on the iodine number or on the horizontal displacements of the "breaks" as these were scarcely altered after draining the acid; thus the undrained sample which gave the points on *ESK*, gave those on *DRJ* after draining.

Consideration of the numerous results obtained makes it possible to conclude that the lower melting modification of pure oleic acid forms with palmitic acid a series of solid solutions, the liquidus "curve"[1] of which is given approximately by the line *AO* (Figs. 1 and 2). Beyond *O*, which corresponds with a total of 7·2 % of palmitic acid and a temperature of 11·8°, another series of solid solutions (liquidus = OF')[2] appears which is doubtless that corresponding with the liquidus curve given by Carlinfanti and Levi-Malvano [1909]. The curve as shown by these authors is the one marked *BC* in Fig. 2, and is such as would be obtained by use of an oleic acid containing some 7 % of palmitic acid and also of other impurities, resulting altogether in a regular displacement of the true curve *AOC* towards the left. The approximate positions of the points on *OC* have been deduced by making regular proportional displacements of *BC* towards the right.

The lowest possible temperature for *O* (Fig. 1) was fixed by that of the break observed with the sample which gave the results indicated by the lines *BP* and *PG* (Fig. 1). This sample had been repeatedly remelted and redrained until its first setting point was constant at 12·95°; it probably contained only minute traces of anything other than oleic and palmitic acids and its "iodine number" was 88·0, corresponding with a content of 2·2 % of palmitic acid.

The true percentage of palmitic acid in the pure eutectic, in other words the position of the point *O* with respect to the left-hand margin of the figure, was decided by reference to the preceding data for the specimen giving the "curve" *BPG*, taken in conjunction with data worked out with special care for a second sample of oleic acid which showed a break of 3·1 % of palmitic acid (added) and which had an "iodine number" of 86·00 (mean of three observations: 86·01, 86·09, 85·76)[1]. The former places *O* at 6·8 % of palmitic acid and the latter at 7·55 %, and *O* has therefore been set about half-way between these points, namely at 7·2 %, with the reservation that this estimate may be inaccurate by 0·5 %.

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[1] These are termed "curves" although within the range of Fig. 1 they did not differ appreciably from straight lines.

[2] When the right-hand branches of the curves were worked out by observations of "first setting points" made with the usual speed of cooling adopted by the authors, namely about 0·2–1·0° per minute, they had slopes roughly parallel with *OF*. This has a slope between that of the true liquidus curve and that of the solidus curve, but considerably nearer to the former. The true liquidus curve is given approximately by the full line OF', which is nearly parallel with the curves which the authors obtained, by plotting the temperatures at which solid disappeared on warming or by plotting "first setting points" when an extremely low rate of cooling was adopted. The slope of *OF* agrees satisfactorily with that of the left-hand extremity of the liquidus curve found by Carlinfanti and Levi-Malvano [1909, p. 371]. In order to make these features clearer the short full line OF'' has been added to the figure; this line indicates the slope (not of course the position) of the left-hand extremity of the solidus curve as given by Carlinfanti and Levi-Malvano.

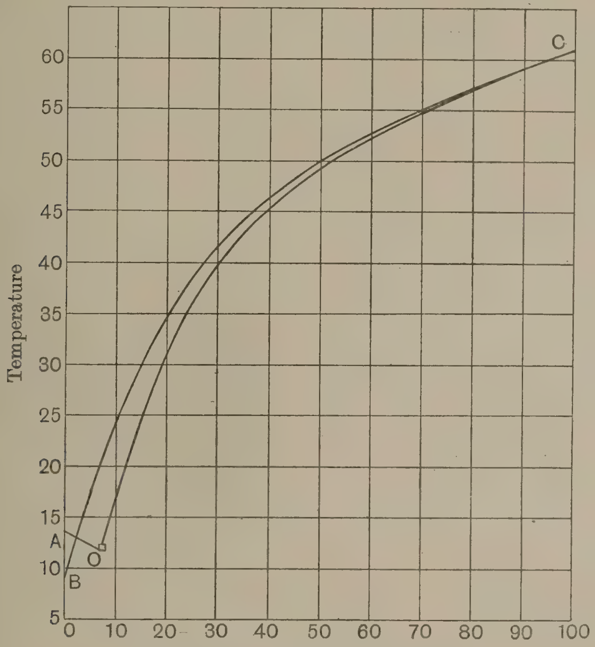

Fig. 2.

The authors believe that any changes in the position of the "curves" *A*, *E*, *B* revealed by more accurate work in the future are not likely to do more than slightly raise the lines *AO* and *OF* (Fig. 1) parallel with themselves or displace them slightly to the right or left. The small square indicates what the authors consider to represent the probable limits of any future alterations in the position of the common point *O*.

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[1] The "iodine numbers" were determined by a modification of the familiar bromine absorption method which was found to give very consistent results. The authors were not wholly satisfied with the bare assumption that the low iodine numbers of the purified samples of acid were due to the presence of palmitic acid, and they therefore oxidised two different samples of acid with alkaline permanganate and isolated from the products that part of it which was soluble in petroleum and non-volatile in steam; it was found to be nearly pure palmitic acid.

Thus: Sample I. Iodine number, 86, corresponding with 95·55 % of oleic acid. Palmitic acid isolated and weighed 3·3 %, making a total 98·85 %.

Sample II. Iodine number 87·2, corresponding with 96·88 % of oleic acid. Palmitic acid isolated, 1·6 %, making a total of 98·48 %.

Taking into consideration the great practical difficulty of isolating the whole of the palmitic acid present in the samples, the authors believe that the results support their view that the "iodine numbers" were trustworthy to 1 part in 100 and that their best samples of oleic acid were free from all but traces of acids of more highly-unsaturated character.

The work described in the present paper was carried out under the auspices of the Oils and Fats Committee of the Food Investigation Board, who defrayed a large part of the expenses incurred.

One of us (L. K. P.) is indebted to the Department of Scientific and Industrial Research for the receipt of a personal grant and another (E. N. M.) for a maintenance allowance as a student receiving training in research.

The authors have pleasure in stating that the work was undertaken at the suggestion of Mr John Allan, whom they have frequently consulted during its progress.

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III. THE NITROPRUSSIDE REACTION OF BACTERIA.

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(*Received November 21st, 1924.*)

In the present investigation an attempt has been made to obtain information as to the presence of glutathione in bacterial cells, as distinct from the culture medium, by means of the nitroprusside test [Hopkins, 1921]. Large quantities of various pure cultures of bacteria were grown in glucose broth, or on agar plates, and then freed from the culture medium. The proteins were removed by precipitation and the filtrate was investigated for the nitroprusside reaction by a quantitative method. Absence of the nitroprusside reaction indicates the absence of glutathione, but the positive reaction cannot be taken as final proof of the presence of this substance until more evidence is available.

Preliminary experiments on the nitroprusside reaction of bacteria were performed by one of us (A. B. C.) in 1922. At that time no quantitative method had been described, so the following method was devised. Pure cultures of bacteria were grown in glucose broth in Roux bottles. The broth was removed by centrifugalisation and the bacteria were washed twice with distilled water, and dried in a vacuum desiccator. The dried mass was powdered finely, and a weighed amount was mixed with 10 cc. of a saturated solution of ammonium sulphate and filtered. All the bacteria examined gave a clear colourless filtrate. In each case 0·2 cc. of a freshly prepared 5 % solution of sodium nitroprusside and 0·2 cc. of strong ammonia were added to the filtrate. The colour obtained was at once compared with a deep pink standard colour. If the filtrate gave a pink colour paler than that of the standard, a larger weight of bacteria was used, and in this way the weight of each organism required to give the standard colour was obtained.

Table I. *Preliminary Experiments.*

	Weight of dried organism which gave standard colour under standard conditions g.
B. pyocyaneus	0·04
B. prodigiosus	0·08
B. proteus	0·1
B. alcaligenes	0·1
B. fluorescens	0·1
Staphylococcus aureus	negative
Sarcina aurantiaca	,,

The results of this experiment show that *Staphylococcus aureus* and *Sarcina aurantiaca* were negative, that is to say, that even large weights failed to give a pink as deep as the standard colour. On the other hand, the other bacteria all gave strong nitroprusside reactions. If the substance responsible for this reaction is glutathione, the actual percentage calculated as dry weight works out as 0·5 % for *B. pyocyaneus*, 0·25 % for *B. prodigiosus* and 0·2 % for the other three positive bacteria. These figures were obtained by matching the standard colour with a known solution of reduced glutathione, which had been treated with nitroprusside as described above.

Since the completion of the preliminary experiments, Tunnicliffe[1] has described a quantitative method for the estimation of glutathione in muscle. Tunnicliffe found that the SH group of reduced glutathione is quantitatively oxidised by iodine. He precipitated the proteins of muscle with trichloroacetic acid and titrated the filtrate with $N/100$ iodine, using the nitroprusside test as an outside indicator. We tried to apply this method to bacteria. *B. alcaligenes* was grown for fifteen hours on forty large agar plates, then carefully scraped off, and ground in a mortar with distilled water until an even emulsion was obtained. 1 cc. was placed in a small weighed centrifuge tube, centrifugalised to remove excess water, then dried in a vacuum desiccator and weighed. In this way, the dry weight of bacteria in each cc. of emulsion was obtained. Another measured volume of the bacterial emulsion (usually 10 or 20 cc.) was precipitated with an equal volume of 20 % trichloroacetic acid. The mixture was ground in a mortar and then quantitatively transferred to a Buchner funnel, and filtered on a pump. The precipitate was removed, washed with distilled water, and again filtered. The two filtrates were added together and titrated with 0·00888 N iodine from a micro-burette, using the nitroprusside test as an outside indicator. Other bacteria were not so easy to manipulate, and solid trichloroacetic acid had to be used for precipitation. With *B. proteus* and *Sarcina aurantiaca*, filtration was so slow that it had to be abandoned in favour of centrifugalisation, the deposit being washed with distilled water, and the washings added to the liquid to be titrated. Several organisms, such as *S. acidi lactici* and the anaerobe *B. sporogenes* were grown on glucose broth instead of on agar plates, in order to obtain heavier growth. In all cases young cultures were used. The dry weight of the bacteria used for titration, calculated from the weight of organisms in 1 cc. of emulsion, is given with the results to show that negative results were not due to lack of material. From the titration result, the number of milli-equivalents of iodine required for 1 g. of each organism (dry weight) was calculated.

The results show that *B. alcaligenes* and *B. proteus* gave a strong nitroprusside reaction, compared with which the other bacteria examined were practically negative. This means that a drop of the trichloroacetic acid filtrate of the latter gave no nitroprusside reaction. If, however, 5 or 10 cc. of the filtrate was examined, most of the bacteria gave a very faint nitroprusside

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[1] In the press.

Table II. *Trichloroacetic Acid Method.*

	Dry weight of bacteria used g.	Number of milli-equivalents of iodine for 1 g. bacteria (dry weight)
B. alcaligenes	1·5072	0·019
,,	1·5885	0·018
B. proteus	0·3577	0·018
,,	0·5890	0·020
B. coli	0·7260	0
B. sporogenes	0·462	0
Streptococcus acidi lactici	0·9240	0
Sarcina aurantiaca	0·2639	0
Staphylococcus aureus	1·1235	0
Timothy Grass bacillus	0·7935	0

reaction. Since very small amounts of glutathione give the reaction, the amount of this substance present in these bacteria must be considered practically negligible. Even when 10 cc. of the filtrate were examined, the Timothy Grass bacillus gave no trace of the nitroprusside reaction, and it is possible that the fatty envelope of this organism may have prevented the escape of any glutathione that might be in the cell. Possibly also in the case of the other negative bacteria glutathione might be present in the cells and yet escape detection, but this is unlikely since the organisms were ground up with trichloroacetic acid. The negative result obtained for the anaerobe *B. sporogenes* agrees with McLeod and Gordon's observation that the anaerobe *B. welchii*, when scraped off the surface of a solid medium, gave no nitroprusside reaction [McLeod and Gordon, 1924].

Three strongly pigmented bacteria, *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus*, could not be investigated by Tunnicliffe's method because in each case the pigment interfered with the titration. The earlier experiments had shown that, when these organisms were precipitated with saturated ammonium sulphate, a colourless filtrate was obtained, so that this method of precipitation was now used in conjunction with the iodine titration. Control experiments showed that the sample of ammonium sulphate used contained no substance capable of reacting with iodine. However, an objection to the use of this reagent is that, although the saturated solution is acid, after being ground up with bacteria the resulting mass is not acid enough to ensure the stability of the SH group. Tunnicliffe and Dixon [1923] showed that the SH group autoxidises far more rapidly in neutral than in acid solution. In order to lessen the possibility of such a decomposition, a trace of sulphuric acid was added to the ammonium sulphate filtrate. Even with this precaution, however, this method has given us slightly lower results than the trichloroacetic acid method. A comparison of the two methods gave the following results for *B. alcaligenes*:

Trichloroacetic acid method: 0·020 milli-equivalents of iodine per g. of dried bacteria.

Ammonium sulphate method: 0·016 milli-equivalents of iodine per g. of dried bacteria.

In default of a better method, the highly-pigmented bacteria were examined by this method.

Table III. *Saturated Ammonium Sulphate Method.*

	Dry weight of bacteria used g.	Number of milli-equivalents of iodine per g. of bacteria (dry weight)
B. pyocyaneus	0·8586	0·019
B. prodigiosus	0·5475	0·018
B. fluorescens	0·6556	0·014
B. coli	0·6928	0

The results show that the three highly-pigmented bacteria, like *B. alcaligenes* and *B. proteus*, contain a considerable amount of the substance responsible for the nitroprusside test. If this substance is glutathione, the percentages can be worked out by Tunnicliffe's experimental data. He found that 1 cc. of $N/100$ iodine reacts with 0·0025 g. reduced glutathione. This gives a value of about 0·47 % for the glutathione content of *B. alcaligenes*, *B. proteus*, *B. pyocyaneus* and *B. prodigiosus*, and 0·35 % for *B. fluorescens*. It will be noticed that these results agree fairly well with those of the preliminary experiments. In each case the same five bacteria gave strong nitroprusside tests, and *Staphylococcus* and *Sarcina* were negative.

At this point we should like to emphasise the fact that at present we have not enough evidence to state that the nitroprusside reaction of bacteria is due to the presence of glutathione, since certain substances other than glutathione also give this reaction. Besides various substances which do not contain sulphur, cysteine, which like glutathione contains the SH group, gives the nitroprusside reaction. Also H_2S in dilute solution gives the pinkish-purple colour that is characteristic of reduced glutathione, although with stronger solutions a deep blue colour is obtained. For this reason, any nitroprusside test performed in the presence of H_2S cannot be considered as evidence of the presence of glutathione.

McLeod and Gordon[1] tested broth cultures of various bacteria for the nitroprusside reaction, but, except in the case of *B. welchii*, they do not appear to have freed the bacteria from the culture medium before performing the test. An obvious objection to this method is that broth cultures of many bacteria contain large quantities of H_2S. However, McLeod and Gordon consider that their positive results are not due to this substance, because the reaction was little, or not at all, reduced by prolonged boiling.

Our method of obtaining bacteria free from H_2S was, firstly, to separate them from the culture medium. Another precaution adopted was the addition of glucose to the broth, as we found that this appeared to lessen or inhibit the production of H_2S by certain aerobes. Moreover, in our experiments, after precipitation of the proteins, the strongly acid filtrate was allowed to boil under reduced pressure on the water pump, and in this way any trace of H_2S would be removed. Proof that this was actually the case is supplied by the results obtained for the anaerobe *B. sporogenes*. This organism formed large quantities of H_2S when grown on glucose broth, and traces of H_2S still remained even after the bacteria had been freed from broth and washed. Yet the trichloroacetic acid filtrate of this organism gave no trace of nitroprusside reaction. Further proof of this point was obtained in the following way. *B. alcaligenes* was grown on agar, the bacteria were scraped off the plates, precipitated with trichloroacetic acid and filtered. Two equal portions of the filtrate were placed in small flasks. Through one of these (Flask A) a stream of nitrogen was bubbled for two hours, the other (Flask B) being placed beside it as a control. Both solutions were then titrated with iodine with the following result.

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[1] Practically all the experiments described in this paper had been completed before the publication of McLeod and Gordon's paper.

Titration result.

Flask A (after nitrogen)	...	2·66 cc. 0·00888 *N* iodine.
Flask B (control)	... ...	2·62 ,, 0·00888 *N* ,,

If the nitroprusside test had been due to H_2S Flask A would have given a lower result, as some of the H_2S would have been removed by bubbling nitrogen through this acid solution. Since the results are practically identical, H_2S cannot be responsible for the nitroprusside reaction of *B. alcaligenes*.

Discussion of Results.

It may be noted that the presence of a substance capable of giving the nitroprusside reaction bears no relation to the oxygen uptake [Callow, 1924] of the organisms investigated. Thus, although *S. acidi lactici* and the anaerobe *B. sporogenes*, which lack the nitroprusside substance, both show practically no oxygen uptake, yet *Staphylococcus aureus* and *Sarcina aurantiaca*, which also lack the substance, both take up large quantities of oxygen. An interesting point is that all the bacteria that give the nitroprusside reaction, under the conditions described, show particularly luxuriant growth on ordinary laboratory media; for instance, on the surface of an agar plate, the growth of these bacteria is considerably heavier than in the case of the other bacteria investigated.

McLeod and Gordon [1924] consider that their results show that the positive reaction obtained with certain *broth cultures* is merely due to the fact that these bacteria reduce the oxidised glutathione which they state is present in the uninoculated broth. They further state that they consider that bacteria are not themselves capable of producing glutathione, or substances allied to it that react with nitroprusside. We cannot yet state whether or not bacteria contain glutathione, but our results show that certain bacteria undoubtedly contain a substance which gives the nitroprusside reaction, and which, like glutathione, is soluble in trichloroacetic acid and can be oxidised by iodine. (Further experiments on this point are in progress and will shortly be published.) On the other hand, we can state that our negative bacteria either lack glutathione, or else their glutathione escapes detection by the methods used in this investigation.

SUMMARY.

1. Bacteria were grown in bulk and separated from the culture medium. The proteins were precipitated with trichloroacetic acid or saturated ammonium sulphate, and the filtrate was examined for the nitroprusside reaction by a quantitative method.

2. *B. coli*, *B. sporogenes*, *Sarcina aurantiaca*, *Staphylococcus aureus*, *Streptococcus acidi lactici* and the Timothy Grass bacillus gave practically no nitroprusside reaction and can therefore be considered to lack glutathione, unless this substance is present in the cells and escapes detection by the methods used.

3. *B. alcaligenes*, *B. proteus*, *B. pyocyaneus*, *B. prodigiosus* and *B. fluorescens* gave strong nitroprusside reactions, the iodine equivalents of which are given.

4. The substance which is responsible for the nitroprusside reaction has not yet been identified, but experiments with *B. alcaligenes* show that it is not H_2S.

In conclusion we gladly take this opportunity of thanking Professor F. Gowland Hopkins, F.R.S., for his encouragement and valuable criticism during the progress of this work. One of us (A. B. C.) is indebted to the Medical Research Council for a whole-time grant.

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IV. THE DIAZO REACTION IN URINE.

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The extensive literature on the "diazo reaction" in urine leaves many problems still unsolved. [For literature *v.* Neubauer-Huppert, 1913.] Ever since it was found that some urines gave an orange or red colour in an alkaline solution of diazotised sulphanilic acid there have been periodic attempts to isolate the substance responsible for the reaction, but so far none of these has been quite successful. Only two colour-giving substances have, as far as the writer can find, been isolated from urine and identified. The first was urocanic acid isolated by Jaffé [1874] and later found by Siegfried [1898] in dogs' urine—the dogs probably presenting a rare anomaly of metabolism. The second substance was histidine, isolated in small amount by Engeland [1908] from 40 litres of human urine. Again, in 1922 the writer [Hunter, 1922, 2] isolated 0·1 g. of histidine as monohydrochloride from a litre of measles urine in which it was estimated that there was 0·31 g. present.

Many suggestions have been made as to the cause of the diazo reaction in urine. Clemens [quoted by von Noorden, 1907] claims to have isolated the sodium salt of the substance responsible for the reaction. Others assume it to be due to chromoxy-proteic acids or to ill-defined members of this group of substances [Neubauer-Huppert, 1913]. Weiss [1920] is emphatic that urochromogen is responsible for the diazo reaction, whilst Hermanns and Sachs [1921] consider that an oxidation product of tyrosine is responsible for the reaction in tuberculous patients and hydroxyindoleacetic acid was indicated in a urine from a patient suffering from cancer of the liver.

Ehrlich first applied the reaction to urine but his procedure has been many times modified and this adds to the difficulty of interpreting the findings by his method. Ehrlich's diazo reagent consisted of a mixture of two solutions [Ehrlich, 1883]:

(*a*) 5 g. of sulphanilic acid with 50 cc. conc. HCl made to 1 l. with water.
(*b*) 5 g. of sodium nitrite made to 1 l. with water.

The solution for carrying out the test was made by mixing 50 cc. of solution (*a*) with 1 cc. of solution (*b*).

In performing the test [Erhlich, 1884] a quantity of urine was taken and an equal quantity of reagent added. If a colour was produced at this stage —what was called a "primary colour reaction"—it indicated the presence of bilirubin. Strong ammonia was then added, and if the test was positive a "secondary colour reaction" was obtained and a red foam on shaking, regarded as of special significance in typhoid, measles, certain types of tuberculosis and other disorders.

There are thus two distinct types of diazo reaction according to Ehrlich—the primary colour reaction in acid solution and the secondary colour reaction in alkaline solution—parallel with the fact that aromatic amines couple with diazonium salts in acid solution and phenols in alkaline solution.

The primary colour reaction of Ehrlich appears to be very specific for bilirubin and this test has recently been used by van Bergh and others [McNee, 1923] in blood serum to distinguish certain types of jaundice. The diazo reaction in acid solution will not be further dealt with in this paper as there has never been any doubt about the substance responsible for it. It is the substances in urine which give a colour with diazonium salts in alkaline solution which have been wholly responsible for differences of opinion in the literature on the diazo reaction in urine; and it is with the diazo reaction in alkaline solution that the writer here purposes to deal. Hitherto only one type of diazo reaction in alkaline solution has been recognised, but, as the sequel will show, there are what may be regarded as two quite distinct types of diazo reaction in alkaline solution.

The writer has had the advantage of using a much more satisfactory diazo reagent than that used by the older investigators on this subject, namely that of Koessler and Hanke [1919], and has reviewed elsewhere the main substances which give a colour with the reagent [Hunter, 1922, 1]. These include phenols, iminazoles, certain purines, tyrosine, sulphides and ammonium salts. The reagent is thus very unspecific but no less so than the older reagents. With careful handling of the solution to be tested the non-specificity of the reagent is perhaps more of an advantage than a disadvantage as it permits the use of a very delicate reagent in the estimation of a wide variety of substances. Its reaction in urine will be considered here only from a qualitative aspect.

The diazo reagent, according to Koessler and Hanke [1919], consists of two stock solutions:

(*a*) 9·0 g. of sulphanilic acid with 90 cc. of 37 % HCl made to 1 l. with water.

(*b*) 50·0 g. of 90 % sodium nitrite made to 1 l. with water.

The *reagent* is prepared by placing in a 50 cc. volumetric flask 1·5 cc. of solution (*a*) along with 1·5 cc. of solution (*b*). After 5 minutes 6·0 cc. of solution (*b*) are added and after another 5 minutes the flask is filled to the 50 cc. mark with cold water. It is recommended by the authors to keep the flask immersed in a freezing mixture, and if kept cold this reagent can be used for at least 24 hours.

A 1·1 % solution of pure anhydrous sodium carbonate is used as alkali.

When the reagent is used for quantitative measurements the sodium carbonate solution and the diazo reagent are first mixed and then the solution to be tested is added.

This preliminary mixing of sodium carbonate and reagent has several advantages, and the mixture remains perfectly clear and colourless for about 30 minutes, so that all the colour developed is due to the test solution added.

The use of sodium carbonate as alkali is also important. Ehrlich used ammonia as alkali although it gives an orange yellow colour with the reagent in a few seconds. On the other hand, Pauly [1904] used solid diazobenzene sulphonate and sodium carbonate, but this mixture quickly turns orange.

Another important point emphasised by Koessler and Hanke is the order in which the solutions are used. The acid reagent has an excess of nitrous acid and may partially deaminise the substance to be tested before it has time to couple in the solution subsequently made alkaline. To prevent such a deamination it is necessary that the reagent be made alkaline before the addition of the test solution.

The reagent of Koessler and Hanke gives very little trouble if certain precautions are taken and the writer follows in general their procedure. The continuous use of ice is, however, not always convenient and can be dispensed with if the temperature of the tap water of the laboratory is less than 10°. The sulphanilic acid solution first put into the reagent flask is allowed to come to the temperature of the running water, then the sodium nitrite solution, preferably kept in the ice chest, is added. The mixing of the sulphanilic acid and sodium nitrite is the critical step in the making up of the reagent as diazotisation is a strongly exothermic reaction, so that the solutions should be as cold as possible before mixing. If attention is not paid to this point unsuitable reagents will often result.

When a sodium carbonate solution which has been made up for some time is used as alkali there is an abnormally rapid colour development, a maximum colour of too yellow a tint and of too low an intensity.

The reagent has been used for qualitative purposes in the following manner by the writer: 5 cc. of 1·1 % sodium carbonate are put in a test-tube and 2 cc. of the sulphanilic acid reagent added. The contents are mixed by shaking the tube but without inverting it. If any colour is developed at this stage it is probably due to a dirty test-tube. Within the space of about 1 minute *one drop of urine* is added. One of two distinct phenomena may occur at this stage: (1) *Alkaline diazo reaction, Type A.* In all urines a faint yellow colour is produced which goes on intensifying for the next 3 to 4 minutes. This colour does not generally develop further than a pale orange but may often show predominately a pink tinge and occasionally quite a marked red. (2) *Alkaline diazo reaction, Type B.* In urines from typhoid and measles a bright orange red colour appears in the solution immediately the drop of urine is added, but the colour disappears in a few seconds. (Thereafter there is an intensification of colour due to the substances responsible for Type A reaction.)

1. *Alkaline Diazo Reaction, Type A.*

Some kind of Type A reaction is obtained in all urines. As a rule the more red the colour obtained, the more iminazole is present. Yet it must be emphasised that this method of qualitative examination is a very poor index of the actual amount of iminazole in urine. This is so because of interfering substances in untreated urine. It has previously been shown [Hunter, 1922, 1] that certain purines interfere with colour production, but the presence of uric acid in urine is alone insufficient to account for the great inhibition of colour development which generally prevails. This is clearly seen by a consideration of the 5 urines in Table I. The amount of iminazole present in each was determined by the recent method of Koessler and Hanke [1924], the amounts taken to obtain the readings given below representing 0·05 cc. of original urine. Alongside these are given the approximate readings obtained from 0·05 cc. of untreated urine. The test cylinder in each case is set at 20 mm. and the cylinder containing the histidine colour standard [Koessler and Hanke, 1919] moved to match.

Table I.

Urine No.	Standard cylinder		Test cylinder mm.
	Untreated urine mm.	Prepared urine mm.	
1	4·5	14·5	20
2	6·5	29·5	,,
3	6·5	27·5	,,
4	2·0	4·9	,,
5	1·0	1·7	,,

It will thus be seen that although urines Nos. 2 and 3 show higher readings than Nos. 1, 4 and 5 by both methods, the readings in Nos. 2 and 3 for the untreated urine represent only about $\frac{1}{5}$ of the colour obtainable from the iminazoles actually present in the urines.

On the other hand some urines give a redder colour by this direct method than one would be led to expect from the actual amount of iminazole present. This appears to arise from a decrease in the concentration of the substances inhibiting colour production. Thus the writer [Hunter, 1922, 2] was led to the conclusion that there was an increased excretion of histidine in urine from measles patients through finding a marked diazo reaction of this type, although as shown there were present 310 mg. of histidine monohydrochloride per litre, a not abnormal amount of iminazole as subsequent work has shown. This reaction, it should be noted, was also mistaken for the characteristic reaction given by measles urines before Type B reaction came to the writer's notice. Indeed, there is ample evidence in the literature that this confusion is common. Thus Neubauer and Huppert [1913] quote at least three workers who found a positive Ehrlich's diazo reaction in a fairly large proportion of normal urines. These positive reactions were probably due to the presence of iminazoles and almost certainly not due to the substance which gives the characteristic test in typhoid and measles. For among several hundreds of normal urines the writer has failed to find one which gives a test of the type given in typhoid or measles.

Of course phenols in urine also contribute to the colour given in Type A reaction. Like the iminazoles the colours for phenols take definite if generally shorter times to develop to a maximum. Likewise purines and tyrosine intensify in colour, though even in pure solution the final colour intensity is not proportional to the amount of the purine or tyrosine present. Ammonium salts and sulphur compounds also give yellow colours, and there are probably several chromogens which either yield a colour in themselves or in some way prevent the diazo reagent from coupling with the iminazoles or phenols.

It may be concluded that the development of a colour predominantly red, of the alkaline reaction A type, is due to the presence of iminazole. The red colour may indicate that the iminazole is present in abnormally large amounts or merely that it is present in normal amounts when the conditions in such a urine are favourable for the coupling of the diazonium salt with the iminazole present. Urocanic acid and histidine are the only two iminazoles so far isolated from urine, though there are probably other iminazoles responsible for Type A reactions.

Type A alkaline diazo reaction is likely to remain of little value as a biochemical test. The interpretation is still too uncertain, as the reagent is too unspecific and too easily interfered with to give reliable information even regarding iminazoles. The normal output of iminazoles in the urine is in the writer's opinion not yet definitely settled although the value of Koessler and Hanke's [1924] contribution in this direction is recognised. But apart from this, practically nothing is yet known of the physiological significance of either a high or a low iminazole excretion. Yet it does appear of importance to distinguish a reaction of Type A from a reaction of Type B such as is obtained in typhoid and measles urines, since Type B reaction holds promise of being a valuable test for certain pathological urines.

2. *Alkaline Diazo Reaction, Type B.*

As far as the writer is aware the alkaline diazo reaction Type B has not hitherto been observed. Its characteristic property is the almost instantaneous appearance of a bright orange red colour of only momentary duration. There is never any doubt as to whether the test is positive or not, as would appear to be in the case of a red foam according to Ehrlich's procedure. Within the past two years the writer has found this type of reaction in the urine of upwards of 30 different measles patients, and in about the same number of typhoid patients. It has been found to persist in some typhoid urines for nearly two weeks, but its onset and disappearance have not been sufficiently studied from a clinical standpoint to permit of discussion here.

A differentiation of two distinct types of diazo reaction in alkaline solution has hitherto been overlooked because of two main factors working together:

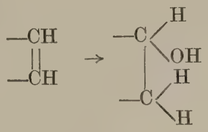
(*a*) with the more concentrated reagents of Ehrlich or Pauly the time of colour development is greatly reduced; (*b*) with the relatively large amounts of urine previously used such an excess of the substance responsible for Type B reaction is in the solution that a fading in colour is not readily observable. Further, the colours produced by iminazoles with the old reagents are less stable than those with the Koessler and Hanke reagents. It also appears that the Ehrlich reagent is less sensitive to the substance responsible for Type B reaction than is the new reagent. Thus, a sample of typhoid urine which showed a beautiful, though momentary, orange red colour when dropped into the new qualitative reagent as described above, when tested by the Ehrlich method showed a bright orange colour quickly, but on shaking, the foam was no more red than that obtainable from a urine containing about 0·04 % iminazole.

There is little doubt that the substance (or substances) responsible for Type B alkaline diazo reaction is new. From the fact that the diazo reaction has always been recognised as occurring in urines, especially from typhoid and measles, it is also most probably true that this substance was responsible for many of the so-called positive diazo reactions recorded in the older literature. Yet the possibility that iminazoles were responsible for many positive reactions can never be quite eliminated. The method suggested for carrying out the test cannot lead to this confusion.

Within the past two years the writer has made various unsuccessful attempts to isolate the substance from both typhoid and measles urines. The substance disappears in a few days from urine which is allowed to become alkaline, but may be kept for a longer period in acid urines. It is not precipitated by neutral lead acetate but is by mercuric acetate. It disappears on treatment with silver nitrate. It is readily soluble in ethyl alcohol and slowly extracted by butyl alcohol from acidified urine. It reduces alkaline potassium permanganate.

No suggestion of the nature of the substance was obtained until the appearance of a recent paper by Baudisch [1924] on the oxidation of pyrimidines by ferrous bicarbonate. (The only substance previously found by the writer to give a Type B reaction was adrenaline.) Baudisch's work was repeated on uracil. One litre of distilled water, 0·01 g. uracil and 5 g. sodium bicarbonate were boiled for 30 minutes in a 2 l. flask. A small test-tube containing 5 g. ferrous sulphate was then suspended in the neck of the flask. A rubber stopper, through which was a glass tube with a cock on the outside, was then tightly inserted into the neck of the flask with the cock open, and the boiling continued for 10 minutes. The flask was then removed from the flame, the cock closed, and the flask cooled under the tap. The flask was then inverted to mix the ferrous sulphate with the sodium bicarbonate solution. White ferrous bicarbonate was thus precipitated. The cock was then opened to admit air, the stopper removed and the contents of the flask shaken in contact with air until all the iron was changed to the red ferric hydroxide. The fluid was then filtered and distilled *in vacuo* at 40° to a volume of about 10 cc. One drop of this solution gave with the diazo reagent a bright purplish colour of only momentary duration. The substance responsible for this test according to Baudisch is an unstable oxidation product of uracil, dihydroisobarbituric acid or a condensation product.

The Baudisch oxidation by ferrous bicarbonate is apparently applicable to cyclic compounds containing an ethylene group. This group is common to the pyrimidines and is oxidised by ferrous bicarbonate by the addition of one molecule of water as follows:



Dihydroisobarbituric acid is unstable and condenses to a yellow-coloured pigment, a bimolecular pinacone-like compound, of an auto-oxidisable nature. The evaporated fluid (dihydroisobarbituric acid or the yellow pigment) reduces ammoniacal silver nitrate solution, gives a blue colour with phosphomolybdic acid and reduces methylene blue.

The writer obtained a reduction with ammoniacal silver nitrate, a blue colour with phosphomolybdic acid and a marked diazo reaction of Type B. The colour obtained with this oxidation product of uracil was however much more purple than that obtained from typhoid urines.

Indole contains an ethylene group like uracil and this was tested as described above for uracil. The final solution gave a reaction of the same type—a momentary orange colour which quickly faded to yellow. This colour was much more like that obtained from the typhoid urines but less bright in colour.

The iminazoles contain an ethylene group; histidine, therefore, was treated in the same manner. The final solution from histidine gave a reaction still of the same type, but the colour was much more yellow than that from typhoid urine. It may be noted here that after treatment with ferrous bicarbonate the solution showed no signs of a diazo reaction of Type A, a finding which shows that the iminazole ring suffers some change.

It cannot be concluded from these findings that the substance in typhoid urine is an oxidation product of indole, merely on the ground that the colour given by oxidised indole is nearer that from the typhoid urine than the other substances tested; for the substance in typhoid urine has not yet been obtained in sufficiently pure solutions to warrant the conclusion that an orange red is its characteristic colour, and, further, there is a wide variety of substances in the body presumably capable of the ferrous bicarbonate oxidation.

It is remarkable that the oxidation products of cytosine, uracil, indole and histidine all give a diazo reaction of Type B. This constancy of finding would suggest that Type B diazo reaction indicates some common atomic configuration such as has been suggested by Baudisch for dihydroisobarbituric acid or the yellow pigment. (Whether it is dihydroisobarbituric acid or its condensation product which is responsible for the reaction does not appear quite clear from Baudisch's paper.) The substance responsible for Type B reaction in typhoid urines has thus probably a part of its molecule of the same configuration as the oxidation products of uracil or of other cyclic compounds with an ethylene linkage. The substance in typhoid urine reduces ammoniacal silver nitrate solution as does the oxidation product of uracil. Both substances are unstable in alkaline solution and reduce alkaline potassium permanganate.

M. Weiss's [1920] conclusion that the substance responsible for the diazo reaction in urine is urochromogen is supported by Baudisch's finding that the oxidation product of uracil condenses to a yellow pigment. It would however seem to be necessary to have a reliable analysis of pure urochrome and also an analysis of the substance in question before the hypothesis of Weiss can be regarded as an established fact.

Further details of the properties of the oxidation products of uracil, or of substances capable of this ferrous bicarbonate oxidation, may suggest new methods for the isolation of this substance from urine. If the substance does not actually prove of importance from a clinical point of view it is at least interesting as a pathological finding and may in time prove to be an exaggeration of some normal physiological process.

Conclusion.

An attempt has been made to systematise the different types of diazo reaction in normal and pathological urines. The Ehrlich diazo reaction in acid solution now used extensively to distinguish different types of jaundice, is only referred to as there has never been any question of its specificity in biological fluids for bilirubin. The diazo reaction in alkaline solution, or more specifically in sodium carbonate solution, has been divided into two types called for convenience Type A and Type B. Type A reaction is characterised by a gradual development of colour until a relatively stable maximum is reached. Type B reaction is characterised by an immediate development and a very rapid disappearance of the colour.

All urines give some form of Type A reaction. The substances in urine mainly responsible for it are iminazoles, phenols, purines and probably unknown chromogens. Type B reaction is given only by pathological urine, especially that from typhoid and measles. The substance responsible for Type B reaction is most probably a hitherto unisolated substance. Some indications of its probable nature have been suggested.

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V. CARNOSINE OF MUSCLE AND IMINAZOLE EXCRETION IN THE URINE.

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One of the conclusions in a recent communication by the writer was that the carnosine content of striated muscle in the cat fluctuates [Hunter, 1924]. It was shown that the carnosine content of white muscle could be lowered; it is now indicated that it can be lowered or raised. Lack of exercise was suggested as a possible cause of the fall in carnosine values shown to occur in certain caged animals. Further work has shown that diet has a definite influence on the carnosine content of muscle, although it has not been disproved that exercise plays a part.

It was previously indicated that a rapid fall in the carnosine content of muscle might be accompanied by an increased iminazole excretion in the urine. This hypothesis appeared true when it was found that two cats, with a high carnosine value in the muscle of an amputated leg, passed, during the following 24 hours—when presumably the muscles were rapidly losing carnosine—urines which showed a more than usually red colour with the diazo reagents.

On the publication of a method for the estimation of iminazoles in urine by Koessler and Hanke [1924] it was decided to test this hypothesis further. The results reported here are evidence in its favour.

The estimation of iminazoles in the urine is of recent date, the first attempt perhaps being made by the writer in 1922 [Hunter, 1922, 1]. It was then shown that a litre of a mixed sample of urine from patients suffering from measles contained 310 mg. iminazole calculated as histidine monohydrochloride, and approximately 100 mg. of the pure substance were isolated and identified.

The recent method of Koessler and Hanke is suitable for the routine estimation of iminazoles in urine. The use by these workers of sodium hydroxide along with lead acetate is more satisfactory than that of baryta as used by the writer. The subsequent evaporation of the filtrate is also indispensable.

Experimental.

1. *Changes in the Carnosine Content of Muscle.*

In Table I is shown the carnosine content of the muscles, gastrocnemius, plantaris and soleus, in the amputated limbs of five cats, and after a varying interval the carnosine content of the corresponding muscles of the opposite limbs. The first date in each case is the day on which the right leg was amputated and the carnosine content of the muscles determined; the second date, the day on which the cat was killed and the carnosine values of the corresponding muscles of the left leg estimated.

Table I.

			Gastrocnemius		Plantaris		Soleus	
Cat No.	Date	Wt. of cat g.	Wt. of muscle g.	% carnosine	Wt. of muscle g.	% carnosine	Wt. of muscle g.	% carnosine
13	26. ii. 24	2681	17·870	0·356	5·184	0·298	3·030	0·051
	2. iv. 24	2710	17·790	0·431	4·859	0·288	3·270	0·050
15	26. iii. 24	1915	13·055	0·131	4·349	0·097	1·710	0·065
	7. iv. 24	1740	11·975	0·023	—	—	1·450	0·064
18	11. vi. 24	2100	13·370	0·014	4·230	0·014	2·212	0·087
	20. vii. 24	3170	22·770	0·258	7·030	0·214	3·500	0·052
			Lateral gastrocnemius					
19	2. vi. 24	3170	9·480	0·386	6·140	0·389	2·890	0·057
	5. vi. 24	2810	8·170	0·166	5·520	0·163	2·900	0·051
			Lateral gastrocnemius					
20	31. v. 24	3168	11·195	0·286	7·519	0·262	3·507	0·082
	12. vi. 24	2750	8·070	0·016	4·534	0·022	3·315	0·084

Cat No. 13 belonged to the same litter as cats Nos. 5 and 6 of a previous paper [Hunter, 1924]. Cats Nos. 5 and 6 gave very low values for the gastrocnemius, viz. 0·01 % and 0·007 % and cat No. 13 would probably have given like values had it been killed at the same time. It was however fed on meat until it was in a moderately good condition. When the right leg was amputated the gastrocnemius contained 0·356 % carnosine. The weight of the cat after the amputation was 2681 g. It recovered well although it lost weight for the following two weeks. A good meat diet was given and the weight increased progressively, reaching 2710 g. when the animal was killed, about 5 weeks after the operation. The weights of the two gastrocnemii were practically the same, but the second carnosine value was 0·075 % greater than the first. This increased carnosine value, however, is not borne out in the values from the plantaris. The soleus values remained, as usual, constant. Owing to the cat's loss of weight during the two weeks following the amputation the carnosine content of the gastrocnemius probably fell during that period.

That the carnosine content of muscle may be increased is clearly shown in the case of cat No. 18. This animal was starved for 15 days during which time its weight fell from 3000 to 2100 g. The carnosine content of the gastrocnemius at the end of the starvation was 0·014 %. For the next three weeks the cat was kept on a maintenance diet of meat and during the following three weeks was given a larger amount so that the weight increased to about 1000 g. more than at the date of amputation. (Further data on this cat are given in Table II.) The carnosine content of the gastrocnemius was now 0·258 %, of the plantaris 0·214 %, and of the soleus 0·052 %. The change in the value from the soleus is unusual.

Cats Nos. 15, 19 and 20 were starved after the operation. In all cases the carnosine values of the white muscles fell.

It may be concluded from the results in Table I that the carnosine content of striated muscle falls during starvation and increases after a meat diet if the weight of the cat increases, even when cats are caged. It is thus clear that if exercise influences the carnosine content of muscle it is not a controlling factor.

2. *Urinary Iminazole of Cats.*

When cats are on a constant diet their daily urine output is fairly regular; and, since they show probably a wider variation in the carnosine content of their muscles than any other laboratory animal, they are peculiarly suitable to use in order to determine whether there is an increased iminazole output in the urine when the carnosine content of the muscle falls rapidly. The iminazole in the urine was estimated according to a modified Koessler and Hanke [1924] procedure. (This modification it is hoped to deal with in detail in a forthcoming paper.) The total daily output of urine was used for each estimation with proportionate amounts of lead acetate and sodium hydroxide. The values are given as "iminazole" according to the suggestion of Koessler and Hanke.

The five cats reported in Table II were all in excellent condition when the urine examination was begun and the carnosine content of their muscles was probably high.

It is notable that with the exception of cat No. 19 the urine on the first day of starvation showed the highest iminazole content over the periods recorded. (Cat No. 21 may be regarded as starving, as it would not at first eat the bread after a diet of meat.) There tends also to be an increased iminazole excretion following amputation, especially so in cats Nos. 19 and 20, where the carnosine value in the muscle is shown to be high. This is less marked in cat No. 18, where the value in the amputated limb was low. The value of 6·0 mg. in this cat following amputation represents two days' output of urine; in such cases the value is generally about double, even if the volume is little changed. This feature is again salient in the same cat on June 14th and 27th, and in cat No. 21 on June 23rd.

The urine from cat No. 17 was not examined over a sufficiently long period to provide conclusive evidence of a fall in iminazole output, as the cat unex-

Table II.

Cat no. 17.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 29	64	12·5	Starvation	Wt. of cat 3520 g.
„ 30	none	—		
„ 31	41	7·8		
June 1	none	—		
„ 2	38	6·8		
„ 3	none	—		
„ 4	47	7·5		1 hour under chloroform
„ 5, 6	none	—		
„ 7	47	6·5		
„ 8	65	9·7		

Cat died on June 9th. Wt., June 9th, 3050 g.
Carnosine: r. plantaris 0·181 %; l. plantaris 0·184 %.

Cat no. 18.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 28	105	12·5	Starvation	Wt. 3000 g.
„ 29	none	—		
„ 30	73	8·2		
„ 31	none	—		
June 1	83	9·4		
„ 2	none	—		
„ 3	57	6·0		
„ 4	35	3·5		
„ 5	49	4·2		
„ 6	none	—		Given 200 mg. carnosine intravenously
„ 7	45	21·5		
„ 8	33	3·2		
„ 9	25	1·2		
„ 10	45	1·3		
„ 11	none	—		Wt. of cat 2100 g.; carnosine 0·014 % (gastrocnemius)
„ 12	78	6·0	90 g. meat per day	
„ 13	none	—		
„ 14	78	10·8		
„ 15	38	8·0		
„ 16	52	7·6		
„ 17	42	4·6		
„ 18	45	4·4		
„ 19	43	4·8		
„ 20	30	4·0		
„ 21	42	4·5		
„ 22	41	5·3		
„ 23	40	5·2		
„ 24	36	4·3		
„ 25	40	3·9		
„ 26	none	—		
„ 27	55	8·5		
„ 28	41	6·1		
„ 29	42	7·0		
„ 30	39	6·1		
July 1	lost	lost		Wt. of cat 2150 g. Given 500 mg. carnosine orally
„ 2	40	7·2		

Cat fed from July 2nd till killed, July 20th, on 200 g. of meat per day.
Wt., July 20th, 3170 g.; carnosine 0·258 % (gastrocnemius).

Cat no. 19.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 27	35	7·9	Starvation ↑	Wt. 3680 g.
,, 28–30	none	—		
,, 31	35	9·0		
June 1–2	none	—		June 2nd, carnosine 0·386 % (lat. gast.)
,, 3	35	14·0		
,, 4	16	4·5		

Cat died June 5th. Wt., June 5th, 2810 g.; carnosine 0·166 %. (lat. gast.)

Cat no. 20.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 31	108	24·2	↑ Starvation ↓	
June 1	none	—		Wt. of cat 3670 g.; carnosine 0·286 % (lat. gast.)
,, 2	140	22·0		
,, 3	none	—		
,, 4	50	7·2		
,, 5	66	6·3		
,, 6	40	3·0		
,, 7	35	3·2		
,, 8	32	3·0		
,, 9	none	—		
,, 10	32	2·2		
,, 11	45	4·5		

Cat killed June 12th. Wt., June 12th, 2750 g.; carnosine 0·016 %. (lat. gast.)

Cat no. 21.

Date	Urine cc.	Iminazole mg.		Diet and remarks
June 14	84	12·7	↑ Bread and water and little milk ↓	
,, 15	157	4·0		Some faeces in urine
,, 16	60	8·0		
,, 17	132	6·5		
,, 18	92	4·2		
,, 19	121	4·5		
,, 20	175	4·8		,, ,, ,, ,,
,, 21	175	3·0		,, ,, ,, ,,
,, 22	none	—		
,, 23	105	7·8		
,, 24	170	7·0		
,, 25	168	4·2		
,, 26	214	5·7		,, ,, ,, ,,
,, 27	150	2·5		
,, 28	80	2·4		
,, 29	181	4·0		
,, 30	98	5·5		
July 1	163	2·5		
,, 2	178	3·6		

Cat kept on above diet till Aug. 4th, when killed. Wt., Aug. 4th, 1860 g.; carnosine 0·023 %.

pectedly died after 10 days' starvation, showing a loss of about 500 g. in weight. At death the carnosine content of the corresponding plantaris muscles was 0·181 and 0·184 %. Even taking account of the irregularity of the urine output the values are relatively high and would probably have fallen after the remaining carnosine had left the muscle.

The iminazole values for cat No. 18 show a fairly progressive fall during the starvation period. For a few days following amputation there is a slight rise in values, after which, for a period of nine days, there is a very regular urine output and the iminazole values show little variation. During the last week of the urine examination the iminazole output again rises owing to an increase in the muscle carnosine following a meat diet.

Cat No. 19 serves to demonstrate a high iminazole output immediately following amputation. This cat also died for unknown reasons after the loss of about 800 g. in weight, the gastrocnemius still showing 0·166 % carnosine. The values as in cat No. 17 are higher than usual throughout.

Cat No. 20 shows a progressive fall in the iminazole of the urine as starvation continues. Over a period of 11 days the carnosine content of the gastrocnemius falls from 0·286 to 0·016 %.

Cat No. 21 was maintained on a diet of bread and water with a little milk added. The urinary output is very high and relatively constant and the iminazole values show some fluctuation. During the first 10 days, however, the values are as a whole higher than in the succeeding period.

During the starvation period, on June 6th, cat No. 18 was given 200 mg. carnosine intravenously. The urine on the following day showed a decided rise in iminazole, representing about one-tenth of the administered carnosine. On July 1st, when on a meat diet, this same cat was given 500 mg. of carnosine sprinkled over the surface of the meat which the cat ate under observation. The urine on the following day showed no marked rise in iminazole output. It is thus clear that a cat can ingest relatively large amounts of carnosine without increase in iminazole output. It is less clear how the cat deals with iminazole actually in the blood stream. For the carnosine was given intravenously at a single injection and at least part of the 21·5 mg. recorded was carnosine as shown by a decrease in colour value on hydrolysis and an increased Knoop test [Hunter, 1922, 2]. However 200 mg. of carnosine is probably not an abnormal amount to be thrown into the circulation on a single day in a cat whose muscle is rapidly losing carnosine—assuming that the carnosine lost by muscle enters the blood stream. For as shown in cat No. 19, the carnosine content of the gastrocnemius fell to the extent of 0·22 % in three days, representing a little over 0·7 g. per kg. of muscle per day. Taking the muscle mass of cat No. 19 as 1400 g. this indicates a loss from the total muscle of rather more than 0·9 g. carnosine. Allowing for red muscle and other muscle with less carnosine than the gastrocnemius, it seems not unlikely that at least 500 mg. may be dealt with by the blood stream per day from this source alone.

The balance of evidence from the urines of the five cats represented in Table II dips in favour of the view that some of the urinary iminazole comes from the carnosine of muscle. Yet there are irregularities in the iminazole excretion which cannot be accounted for on this assumption. Perhaps a clear proof is not to be expected, for, if carnosine is a reserve material, it is probably in large part utilised by the starving animal. Further, when carnosine rapidly disappears from muscle only a fraction may reach the blood stream, and of this fraction only a part of the carnosine or its breakdown products is likely to reach the urine with the iminazole ring intact. An increase in the iminazole content of the blood will require to be demonstrated before it can be determined whether the carnosine lost from muscle enters the blood stream or is directly utilised by muscle. There is not yet a suitable method for the estimation of iminazoles in small quantities of blood. The writer has found by a fractional precipitation that there are at least 1 to 2 mg. of carnosine per 100 cc. of ox blood.

An attempt was made in several of the cat urines to determine the main iminazole present. In one cat the lead acetate filtrates were collected and precipitated by mercuric acetate. Nearly all of the colour giving substance was precipitated in this manner. The substance was also precipitated in a *slightly acid solution* by silver nitrate. The final solution obtained after several precipitations in this manner gave a colour which developed more quickly and was finally more red than that obtained from histidine. The solution of the substance was strongly acid to litmus and rapidly decolorised alkaline permanganate. It gave a strongly positive Knoop test.

Its behaviour in the silver precipitation, its acid reaction, and its unsaturation parallel the properties of urocanic acid. It is not stated in the literature whether urocanic acid gives a positive Knoop test and its colour with the modern diazo reagents has not been compared with that obtained from histidine.

It was estimated on an arbitrary molecular colour value of 130 million mm. that there was about 0·2 g. of the substance in the solution above referred to, yet crystals could not be obtained directly in the solution, nor with picric or nitric acids. It might be supposed from the literature on urocanic acid that at least a picrate should have been obtained under the conditions were the substance urocanic acid[1].

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[1] Through the courtesy of Professor Andrew Hunter of this University the writer has obtained some pure urocanic acid. A saturated solution of this substance (about 0·2 % at 20°) is quite negative to Knoop's test. Its behaviour with the diazo reagents is also noteworthy. A solution containing 0·04 mg. per cc. of $C_6H_6O_2N_22H_2O$, when tested for histidine and carnosine, as described in the paper which follows, gives a molecular colour value of approximately 109 million mm. The final colour developed by urocanic acid with the diazo reagents is, however, more yellow than that produced by histidine and its time of colour development is much more prolonged, viz. 12–14 mins. The intensity of colour produced is proportional to the amount of urocanic acid present.

Although the substance from cat's urine above described is acid in reaction, unsaturated and gives a colour like iminazoles with the diazo reagents, it is most probably not urocanic acid because it is much more soluble in water, it gives a Knoop test and behaves differently with the diazo reagents as regards time of colour development and shade of colour.

SUMMARY.

1. The carnosine content of striated muscle in the cat can be lowered by starvation, and if lowered can again be raised by a meat diet.

2. When carnosine disappears rapidly from muscle there is evidence of an increased excretion of iminazole in the urine.

Acknowledgment is due to the Medical Research Committee, University of Toronto, for the animals used.

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VI. COLOUR STANDARDS FOR USE IN THE DETERMINATION OF IMINAZOLES.

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(*Received December 8th, 1924.*)

The use of a mixture of Congo red and methyl-orange solutions was recommended by Koessler and Hanke [1919] when these workers published their method for the determination of iminazoles. Suitable mixtures of the two pigments gave colours which matched closely the colour developed by mixing solutions of histidine or other iminazole with the diazo reagent in a sodium carbonate solution.

It is a good rule in colorimetric work to use a standard made from the substance to be measured rather than to employ an artificial standard; but in the case of iminazoles the use of an artificial standard is almost indispensable, chiefly because it is impracticable to make up the standard and test solutions exactly at the same time, a procedure which the relative instability of the colours obtained would seem to demand. Further, iminazoles are as a rule difficult to prepare, and with the exception of histidine are not on the market. A reliable artificial standard will thus always serve as some check on the purity of iminazole solutions of different workers.

The stock solutions of Congo red and methyl-orange recommended by Koessler and Hanke [1919] were stated to keep indefinitely. This seems to be true. Recently, however, mainly to test this point, the writer had occasion to revise his standards. Previously, vacuum dried Grübler's Congo red and methyl-orange had been used as recommended originally by Koessler and Hanke. In this instance it was thought desirable to recrystallise the Grübler products and compare the standard made from these with the old standard. The new standard was found to have about 25 % greater colour value than the old standard. This finding was not, as at first suspected, due to a diminution of colour in the old stock solutions through standing, but was due to impurities in the Grübler's Congo red and methyl-orange.

The Congo red of four different manufacturers was tested and ranged from 40 % to 75 % of the colour value of the purified Congo red. Different methyl-orange products showed a like range of variation.

This difference in colour value is borne out by the residues obtained on ignition from the purified and commercial products. Both Congo red and methyl-orange are sodium salts, and thus leave a residue on ignition. The theoretical amount of sodium, weighed as sodium sulphate, can be calculated from the formulae. Thus Congo red, $C_{32}H_{22}O_6N_6S_2Na_2$, contains 6·62 % of sodium or 20·44 % as sodium sulphate. The formula of methyl-orange is $C_{14}H_{14}O_3N_3SNa$, which yields 21·71 % sodium sulphate.

Before dealing with the ash values the method of purification of the pigments will first be given.

1. *Congo red.* About 10 g. of Grübler's Congo red with 150 cc. of water are heated in a beaker placed in a boiling water-bath and stirred until the sediment is at a minimum, when the contents of the beaker are filtered through a Buchner previously heated by steam. The filtrate is reheated on the water-bath and 150 cc. of 95 % alcohol added. It is then placed in an ice chest over night. The crystal mass is filtered by suction, washed with alcohol, and allowed to dry in the open. The yield (about 7 g.) is ground in a mortar, redissolved in 100 cc. of hot water, filtered and reprecipitated by an equal volume of alcohol. The final air dry product is ground and heated in a water oven until constant in weight, when it is assumed to be water free: Yield 4–5 g.

2. *Methyl-orange.* A beaker containing 200 cc. water and 15 g. of Schuchardt's methyl-orange, which was found to give a rather higher colour value than Grübler's, is placed in a boiling water-bath. The solution is filtered through a hot Buchner, and the filtrate allowed to cool, when the methyl-orange immediately separates out. The solution is allowed to stand at 0° overnight. The methyl-orange is then filtered off, washed with cold water, and dried in the open. About 10 g. of dry material are then redissolved in 100 cc. of water and the procedure, as described, repeated. The final air dry product is ground and brought to constant weight in the water oven. A final yield of almost 8·0 g. is obtained.

It is advisable to keep the substance in an evacuated calcium chloride desiccator rather than in one containing sulphuric acid, as Congo red will darken in colour in presence of the acid.

The residue on ignition as sodium sulphate is perhaps the most convenient index of the degree of purity of Congo red or of methyl-orange. The only disadvantage of ashing is that a little of the sodium appears to volatilise on combustion of the carbon before the addition of the sulphuric acid.

The following percentages of sodium sulphate were found in Grübler's products and in the Congo red and methyl-orange twice recrystallised. The high residue on ignition from Grübler's Congo red is due mainly to sodium sulphate and from the methyl-orange to sodium chloride.

	Congo red % Na_2SO_4	Methyl-orange % Na_2SO_4
Grübler's	41·95	49·40
Twice recrystallised	19·42	20·45
Theoretical	20·44	21·71

The above findings necessitate a new basis for the making of the colour standards used in the estimation of iminazoles. If the quantities of stock Congo red and methyl-orange recommended by Koessler and Hanke are taken from pure solutions of these pigments the molecular colour values of the different iminazoles are materially lowered and a correction of all the work previously done is required. This, however, is unnecessary if a new colour standard is made to give a molecular colour value of 114 million mm. for histidine—the value originally given by Koessler and Hanke. The writer [Hunter, 1921] previously found a rather higher molecular colour value for histidine so that his colour standard was probably more dilute than that used by Koessler and Hanke. For this reason the molecular colour value of carnosine is now appreciably lowered. This does not affect the writer's results previously published for carnosine as the molecular colour value was accurate according to the colour standard then used.

The stock solutions are made as follows:

Stock methyl-orange. This consists of 0·1 g. of pure methyl-orange made to 100 cc. with water.

Stock Congo red. This consists of 0·2 g. of pure Congo red made to 100 cc. with water.

Koessler and Hanke originally recommended 0·5 % Congo red, but with pure Congo red this is too concentrated for accurate pipetting. These authors also used 10 cc. of alcohol per 100 cc. This appears quite unnecessary as Congo red is more soluble in water than in alcohol. Alcohol, indeed, gives the solution a turbid appearance for at least some days after mixing.

Histidine Colour Standard (*S*). About 80 cc. of distilled water are taken in a 100 cc. flask and 0·40 cc. stock Congo red added. After mixing, 0·06 cc. stock methyl-orange is added and the flask filled to the mark with water.

This colour standard, which contains a rather greater proportion of Congo red than that originally recommended by Koessler and Hanke [1919], matches excellently the colour produced by histidine and at the same time keeps the molecular colour value of this substance at about 114 million mm.

The colour produced by carnosine is slightly redder than that obtained from histidine but may be easily read against the above histidine colour standard. For this reason the writer has discontinued the use of a separate colour standard of carnosine.

The molecular colour values of histidine and carnosine were determined as follows: aqueous solutions were made containing 0·03 mg. histidine, 0·05 mg. histidine monohydrochloride, 0·03 mg. carnosine and 0·05 mg. carnosine nitrate per cc. respectively.

From each of the four test solutions thus prepared five readings were obtained using amounts differing by 0·2 cc. The reading given by 0·01 mg. of the substance is thus obtained and the molecular colour values calculated as shown below. In each case the molecular weight × the reading for 0·01 mg. × 10^5 = the molecular colour value in mm.

1. *Solution containing* 0·03 *mg. histidine per cc.*

Amount of test solution used cc.	Colour standard reading, S mm.	Test solution reading, T mm.
1·0	22·1	20
0·8	17·5	,,
0·6	13·2	,,
0·4	8·7	,,
0·2	4·4	,,

From these readings 0·01 mg. histidine gives a reading of approximately 7·4 mm.; whence the molecular colour value of histidine is

$$155 \times 7{\cdot}4 \times 10^5 = 114{\cdot}7 \text{ million mm.}$$

2. *Solution containing* 0·05 *mg. histidine monohydrochloride per cc.*

Amount of test solution used cc.	Colour standard reading, S mm.	Test solution reading, T mm.
1·0	26·8	20
0·8	21·5	,,
0·6	16·2	,,
0·4	10·8	,,
0·2	5·4	,,

From these readings 0·01 mg. histidine monohydrochloride gives a reading of approximately 5·4 mm.; whence the molecular colour value of histidine is

$$209{\cdot}5 \times 5{\cdot}4 \times 10^5 = 113{\cdot}1 \text{ million mm.}$$

3. *Solution containing* 0·03 *mg. carnosine per cc.*

Amount of test solution used cc.	Colour standard reading, S mm.	Test solution reading, T mm.
1·0	18·6	20
0·8	15·0	,,
0·6	11·1	,,
0·4	7·4	,,
0·2	3·8	,,

From these readings 0·01 mg. carnosine gives a reading of approximately 6·2 mm.; whence the molecular colour value of carnosine is

$$226 \times 6{\cdot}2 \times 10^5 = 140{\cdot}1 \text{ million mm.}$$

4. *Solution containing* 0·05 *mg. carnosine nitrate per cc.*

Amount of test solution used cc.	Colour standard reading, S mm.	Test solution reading, T mm.
1·0	24·8	20
0·8	19·5	,,
0·6	14·6	,,
0·4	9·8	,,
0·2	4·9	,,

From these readings 0·01 mg. carnosine nitrate gives a reading of approximately 4·9 mm.; whence the molecular colour value of carnosine is

$$289 \times 4{\cdot}9 \times 10^5 = 141{\cdot}6 \text{ million mm.}$$

The molecular colour value of histidine as determined from the free base and from the monohydrochloride differs by about 1 %. It may be taken at 114 million mm. as measured by the new standard.

With the same colour standard the molecular colour value of carnosine as determined from the free base and from the nitrate is 141 million mm.

SUMMARY.

It has been shown that the best commercial Congo red and methyl-orange are not suitable, without purification, for use as artificial standards in colorimetric work.

A colour standard made from pure Congo red and methyl-orange suitable for the estimation of histidine or carnosine is described.

The purity of other dyes used for colorimetric standards should be ascertained before standardising against particular iminazoles, phenols or other substances measurable by the diazo method.

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VII. THE PRESENCE OF VITAMIN A IN YEAST FAT.

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(*Received December 12th, 1924.*)

It is well known that yeast is comparatively rich in vitamin B and is frequently used as a source of this substance. The evidence as to the existence of the fat-soluble A vitamin in yeast is however not so clear and the presence of this accessory factor in yeast does not seem to be generally recognised.

Hopkins [1912], in his classic paper on the importance of accessory factors in normal dietaries, described experiments which certainly indicate the presence of the A vitamin in yeast. In these experiments rats were fed on a basal diet consisting of casein, starch, sugar, lard and salts; the addition of 4 % of milk to this dietary induced normal and continued growth. Protein-free alcoholic extracts of milk-solids and of yeast in very small amounts produced a similar acceleration of growth; it is also stated in the same paper that a minute amount of the ether-soluble fraction of the total alcohol extract of yeast induced great acceleration of growth. The significance of this observation seems to have escaped general notice. The diet used was deficient in the three accessory factors A, B and C, and the addition of yeast to the diet would certainly furnish an adequate supply of the B vitamin. It is improbable however that the ether-soluble fraction of the alcohol extract of yeast contained any significant amount of the B vitamin since as a general rule the B vitamin is not extracted by ether [Medical Research Committee, 1924]. It seems almost certain therefore that the acceleration of growth obtained in these experiments was caused by the addition of the ether-soluble A vitamin. Macallum [1919] also found that the addition of 2 or 6 % of dried brewer's yeast to a diet deficient in vitamins A and B induced satisfactory growth in rats for short periods of time.

It seemed to us desirable to investigate directly whether acceleration of growth in rats is produced by the addition of yeast fat (or more accurately, of the ether-soluble constituents of yeast) to a diet which already contained a supply of the B vitamin. In this case if the yeast fat was effective in inducing growth, the presence of the A vitamin in yeast would be satisfactorily confirmed.

A study of yeast fat by one of us [Smedley MacLean, 1922] has shown that the percentage of fat in yeast may be considerably increased by incubating the yeast in a solution of sugar, such as fructose, glucose or sucrose. It is difficult however to obtain the increased amount of fat by directly extracting the dried yeast with ether; on the other hand, it is quite easily extracted if the yeast is first boiled for two hours with a normal solution of hydrochloric acid. The carbohydrate of the yeast is hydrolysed and both carbohydrate and protein pass into the acid solution, the fat being readily extracted from the dried residue. A comparison of the A vitamin content of the yeast fat obtained by direct extraction with that of the fat obtained from the yeast residue after hydrolysis of the carbohydrate was undertaken. It seemed of interest to determine whether the conditions which led to an increase of fat in the yeast cell also led to an increase of the A vitamin.

Almost all the additional fat formed when the yeast is incubated in a sugar solution is contained in the fat extracted after treatment of the yeast with the normal acid solution; it was therefore interesting to determine whether such a sample of fat contained the A vitamin or whether this vitamin was contained almost entirely in the first direct ether extract.

The experiments were carried out according to the method recommended by Zilva and Miura [1921] and the basal diet was that used by these observers containing the B and C vitamins but free from A vitamin. It consisted of a mixture of casein 20 %, starch 50 %, salts 5 %, hardened cottonseed oil 15 %, marmite 5 %, and decitrated lemon juice 5 %. After the usual preliminary period on the basal purified diet, indicated by dotted lines in the growth curves (Figs. 1, 2 and 3), the various substances to be tested were given to the animals daily for 28 days. The calcium content of the bones of both hind legs (femur, tibia and fibula) was estimated in each case by Aron and Sebauer's method [1910].

The results of these experiments are shown in Figs. 1, 2 and 3. The rats in each group are of the same sex and taken from the same family.

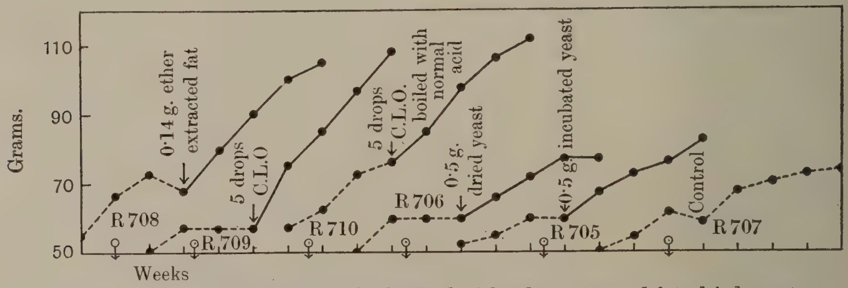

Fig. 1. A comparison of growth obtained with ether extracted fat, dried yeast, incubated yeast, and maximal doses of cod-liver oil.

% Ca in dry weight of bones

R 707	16·2	R 710	19·4
R 705	16·1	R 709	19·1
R 706	15·8	R 708	18·1

In Fig. 1 comparison is shown of the growth obtained with daily doses (*a*) of fat obtained by directly extracting dried yeast with ether (0·14 g.); (*b*) of dried yeast (0·5 g.); (*c*) of yeast incubated in carbohydrate solution to increase the fat content and then dried (0·5 g.); and (*d*) of cod-liver oil (5 drops daily, about 0·14 g.). The results show absence of growth obtained with both specimens of dried yeast, positive growth with the yeast fat and with the cod-liver oil.

In Fig. 2 the results are given of the tests with the fat obtained by direct extraction of the dried yeast with ether and with the fat obtained from the residue by boiling it with acid and then drying it and extracting it with ether. In the first case there was growth, in the latter not.

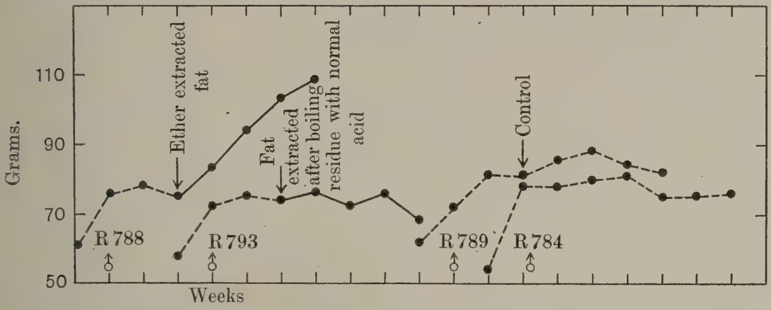

Fig. 2. A comparison of growth obtained with ether-extracted and hydrolysed fat from the same yeast.

% Ca in dry weight of bones

R 784	12·6	R 793	14·7
R 789	14·2	R 788	16·3

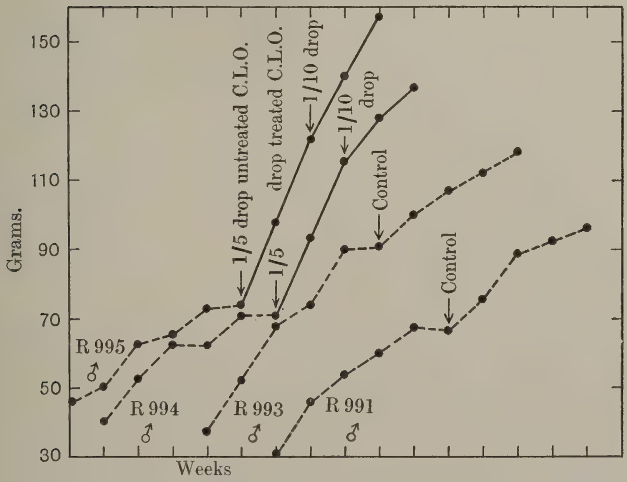

Fig. 3. Test for the destruction of the growth factor in cod-liver oil by boiling with normal acid (minimal dose).

R 991	17·8	R 994	18·7
R 993	16·1	R 995	21·9

As far as these experiments go they indicate that the A vitamin is readily extracted by ether with the more easily obtained fat and there is no evidence that the residual fat is associated with the A vitamin.

Tests were then made to determine how far the process of hydrolysis used had a destructive effect on the A vitamin. A sample of cod-liver oil was submitted to the same process of boiling with normal acid and the effect of the oil thus treated compared with that of the original sample. If a dose of 5 drops of each of the two samples was given daily no difference could be detected in their results (Fig. 1), but with a minimal dose the effect becomes apparent. Fig. 3 shows that with a minimal dose of $\frac{1}{10}$ of a drop of oil there was some destruction of the growth factor in the sample that had been heated with the acid. The growth of rat 994 ran parallel with that of 995 until the dose was reduced from $\frac{1}{5}$ to $\frac{1}{10}$ of a drop.

DISCUSSION OF RESULTS.

The negative results obtained when dried yeast was added to the basal diet (Fig. 1) are probably explained by the dosage. Dried brewer's yeast used in these experiments contains about 3 % fat and 0·5 g. dried yeast contains therefore only about 0·015 g. of fat, a quantity insufficient to maintain growth; the dried yeast after incubation contains three or four times as much fat as the original sample but we have been unable to obtain evidence that there has been any corresponding increase in the growth factor. It was difficult to feed large doses of the dried yeast and the dosage is more satisfactorily tested by giving the ether-soluble constituents; the results obtained when a dose of 0·15 g. of the yeast fat was administered daily (Fig. 1) show that the growth factor is present in yeast; an increased calcium content is also found on analysis of the bones.

The striking difference in the results obtained when (*a*) the fat directly extracted by ether and (*b*) the fat dissolved by ether after the residue has been treated with acid shows that the A vitamin probably occurs in the cell in an easily extractable form and is not associated with the residual fat which is more firmly retained in the cell; we did not obtain any indication that increase of vitamin accompanied the increase of fat which occurs when the yeast is incubated in a sugar solution.

The yeast used in these experiments was ordinary brewery yeast grown in the absence of direct sunlight, the surface of the wort only being exposed to diffuse light. The question arises as to whether the yeast was able to synthesise the fat-soluble vitamin in the absence of any direct solar radiation or whether this accessory factor was picked up by the yeast from the medium and concentrated in the yeast.

Since the wort in which the yeast is grown consists essentially of an aqueous extract of malted barley with or without the addition of carbohydrate, if the fat-soluble vitamin has not been synthesised by the yeast it must have been preformed in the malted barley.

Steenbock, Kent and Gross [1918] have shown that whereas barley is rich in the water-soluble B vitamin it is deficient in the fat-soluble factor. They found that in feeding experiments where all other requirements had been provided for, the diet of rats must contain 60 % of barley in order to get improved growth with a diet otherwise deficient in the fat-soluble factor.

Southgate [1924], who examined barley, kilned malt, unfined and fined beers for the A vitamin was unable to detect it in any of the materials examined.

From what is known of the solubility of the fat-soluble vitamins it is improbable that the process of macerating the malted grain with water would result in the extraction of any appreciable amount of them. After boiling the wort the almost clear liquid is drawn off and as large a surface as possible of the hot liquid exposed to the air so that efficient aeration may take place, the aeration being continued during the process of cooling. Great importance is attached by the brewer to the thorough aeration of the wort and there is no doubt that this process would be highly detrimental to any A vitamin present in the original liquid.

It seems more probable therefore that the A vitamin is synthesised directly by the yeast cell and that this synthesis is brought about in the absence of any direct solar radiation. This result, if definitely established, would be of interest since, in so many instances, solar energy has been shown to play an important part in the production of substances rich in the fat-soluble vitamin.

Ehrlich [1907] showed that the higher alcohols, which are derived from the amino acids of the nutrient protein, are only formed when carbohydrate fermentation is taking place; it is possible that the energy set free by the fermentation of the carbohydrate enables the yeast cell to carry out various metabolic processes, not the least important of which may be the synthesis of the fat-soluble vitamin.

We desire to express our indebtedness to the Food Investigation Board for a grant which has enabled this investigation to be carried out; to thank Miss D. G. Hoffert for assisting us in the work and to acknowledge the courtesy of Messrs Watney Coombe and Reid in supplying us with material.

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VIII. THE EFFECT OF IRRADIATION AND DIET ON CALCIUM AND PHOSPHORUS METABOLISM.

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(*Received December 15th, 1924.*)

The metabolism of calcium and phosphorus is intimately involved in the action of ultra-violet irradiation. Hess and Lundagen [1922] have shown that in infants there is a seasonal tide of blood phosphate depending on the seasonal variation in sunlight. The increased deposition of calcium and phosphorus in bone as a result of irradiation, whether natural or artificial, has been amply proved by such work as that of McCollum and others [Powers, Park, Shipley, McCollum and Simmonds, 1921]; and numerous experiments have been carried out in investigating the effect of ultra-violet light on growth and the prevention of xerophthalmia.

In most of the above instances, however, the changes in calcium and phosphorus metabolism have been deduced from a final chemical analysis of the tissues, or from histological examination. In only very few cases has a complete calcium and phosphorus balance been followed out.

The present work was accordingly undertaken with the object of obtaining a quantitative estimate of the retention of these minerals under the influence of light over a fairly long period. Previous work, for the greater part, where it has not been on the human subject, has been carried out on small animals. For the present investigation the pig was selected for the following reasons:

(1) The growing pig of about three months old retains large amounts of calcium and phosphorus—roughly about 5 g. of CaO and of P_2O_5 per diem [Orr, 1923].

(2) A considerable amount of data on the retention of these minerals by the pig has already been accumulated at this Institute and serves as a guide.

(3) In the pig there is no thick coat to interfere with irradiation of the skin.

The importance of the proportions in which inorganic constituents are present in the diet has been shown by Elliot, Crichton and Orr in their work on rickets in pigs [1922]. In the case of the rat, Hess, Unger and Pappenheimer [1922] find that the rickets-producing effect of a diet adequate in calcium but low in phosphorus may be prevented by short exposures to direct sunlight. In the present investigation the calcium and phosphorus retention was accordingly determined in irradiated pigs which were on diets ill-balanced and well-balanced respectively, particularly as regards their calcium, phosphorus and magnesium content. This aspect of the problem is of interest since the interrelation between ultra-violet light and vitamin A has already been carefully worked out by Hume [1922], Goldblatt and Soames [1923], Steenbock and Nelson [1923]. The problem, too, is not without its clinical significance in the treatment of rickets [Mellanby, 1924].

Two separate experiments were carried out. In the first, two pigs were used. One was irradiated and one kept in the dark. Both pigs were on a diet badly balanced as regards several of the mineral constituents. In the second experiment three pigs were used; one irradiated, one in darkness and one in diffuse light. All three were on a diet well-balanced as regards its mineral content.

EXPERIMENTAL.

Experiment 1. *Animals.* Two hog pigs of ten weeks old were selected from the same litter. They were closely comparable as regards condition, and at the beginning of the experiment each weighed 21·5 kg.

Housing. The pigs were kept in large metabolism cages from which urine and faeces could be collected separately. All sunlight entering the room traversed glass; further, the cages were rendered practically dark by means of green cloth canopies at a height of 9 inches above each cage and draped down the sides. There was no interference with ventilation. The animals were weighed every morning and were allowed to run about the animal room for half an hour for exercise.

Diet. The ration was as follows:

	g. per day	CaO %	P_2O_5 %	N %
Oatmeal	180	0·0819	1·048	2·101
Maize-meal	270	0·0188	0·526	1·511
Middlings	450	0·1245	1·528	2·261
$CaCO_3$	6	3·8898	—	—
Distilled water	*ad lib.*	—	—	—
Intake per 48 hours	—	8·2300 g.	20·366 g.	36·072 g.

A comparison of the mineral ratios of this diet and of sow's milk shows the diet to be ill-balanced with regard to several of its inorganic constituents. Thus:

	Sow's milk	Diet
$CaO : P_2O_5$	10 : 9	10 : 25
$CaO : MgO$	20 : 1	20 : 19

The content of the ration in fat-soluble vitamin is low.

Analysis. The metabolism cages were washed out with distilled water every morning. The excreta were collected over 48-hour periods and the analyses then carried out. Calcium was precipitated as oxalate and estimated volumetrically. A slightly modified Pemberton-Neumann method was adopted for phosphorus.

Irradiation. The source of light was a carbon arc lamp which took ·36 ampere at 45 volts. The pig was irradiated for 1 hour daily at a distance of 3 feet from the lamp.

The time during which the animals were in the cages may be divided into three periods. (1) A preliminary period of 10 days to accustom them to their environment and to determine their appetites. (2) A pre-period of 10 days during which analyses of the excreta were carried out. (3) An experimental period of 24 days during which the analyses were continued and the irradiation of the irradiated pig was in progress. The condition of the non-irradiated pig later in the experiment became so unsatisfactory that for the last few days an attempt was made to improve it by irradiation.

Forty-four days after the pigs had been put in the metabolism cages they were killed. Autopsies were done and also analyses of the blood and bones.

Table I. Exp. 1. *Average daily excretions and balances in grams.*

				Calcium (intake: 4·11 unless indicated thus *)				Phosphorus (intake: 10·18 unless indicated thus *)			
	Days	Wt. of faeces	Wt. of pig (kg.)	Urine	Faeces	Total	Balance	Urine	Faeces	Total	Balanc
Irradiated pig 1193.											
	1–10	522	21·9	0·09	3·56	3·65	0·46	1·51	6·79	8·30	1·88
Light	10–18	735	22·4	0·14	3·42	3·56	0·55	2·23	6·56	8·79	1·39
	18–24	513	21·7	0·23	1·73	1·96	1·78	2·48	4·68	7·16	2·54
	24–32	614	22·1	0·14	1·63	1·77	2·10*	2·76	4·10	6·86	2·65*
Non-irradiated pig 1192.											
	1–10	488	22·0	0·08	4·02	4·10	0·01	1·31	7·08	8·39	1·79
	10–18	373	22·2	0·10	2·64	2·74	0·26*	1·86	4·98	6·84	0·58*
	18–24	290	22·5	0·10	2·53	2·63	0·35*	1·51	4·38	5·89	1·45*
Light	24–32	457	22·7	0·10	3·02	3·12	0·10*	2·34	5·49	7·83	0·15*

* Owing to pig 1192 latterly refusing to consume the whole ration the average daily intake for the last three periods was 3·00, 2·98 and 3·22 g. CaO; and 7·42, 7·34 and 7·98 g. P_2O_5.

Table II. Exp. 2. *Average daily excretion and balances in grams.*

					Calcium (intake: 11·94 CaO)				Phosphorus (intake: 12·77 P_2O_5			
		Days	Wt. of faeces	Wt. of pig (kg.)	Urine	Faeces	Total	Balance	Urine	Faeces	Total	Balanc
Irradiated pig 1322.												
		1– 8	447	37·5	0·41	6·50	6·91	5·03	0·85	6·69	7·54	5·23
Light		8–16	510	40·2	0·46	7·06	7·52	4·42	0·87	7·12	7·99	4·78
		16–24	397	43·1	0·42	6·12	6·54	5·40	0·60	6·36	6·96	5·81
	C.L.O.	24–32	463	45·2	0·31	6·81	7·12	4·82	0·69	7·14	7·83	4·94
		32–40	391	48·1	0·51	6·32	6·83	5·11	0·88	6·61	7·49	5·28
		40–50	398	51·6	0·56	6·52	7·08	4·86	0·87	6·73	7·60	5·17
Non-irradiated pig—in darkness 1321.												
		1– 8	489	37·1	0·37	7·10	7·47	4·47	1·21	6·90	8·11	4·66
		8–16	459	38·4	0·30	7·77	8·07	3·87	1·40	7·84	9·24	3·53
		16–24	458	42·6	0·26	6·52	6·78	5·16	1·33	7·42	8·75	4·02
		24–32	378	45·1	0·27	6·24	6·51	5·43	1·04	6·53	7·57	5·20
		32–40	420	48·1	0·31	6·47	6·78	5·16	1·00	7·51	8·51	4·26
	C.L.O. last 6 days	40–50	401	51·7	0·21	6·57	6·96	4·98	1·14	6·71	7·85	4·92
Non-irradiated pig—in diffuse light 1323.												
		1– 8	445	33·8	0·51	7·75	8·26	3·68	0·58	7·82	8·40	4·37
		8–16	479	36·2	0·45	7·85	8·30	3·64	0·60	7·63	8·23	4·54
		16–24	423	38·9	0·28	7·10	7·38	4·56	0·78	7·11	7·89	4·88
		24–34	388	42·2	0·29	6·32	6·61	5·33	0·65	6·55	7·20	5·57

Results. Fig. 1 shows the calcium and phosphorus balances for each pig. The abscissae mark days, while the ordinates represent retention of CaO and P_2O_5 in g. per diem. Before irradiation was begun the CaO and P_2O_5 balances were fairly steady, the CaO having shown a slight decrease. Comparing the experimental with the pre-period in the irradiated animal the following points may be noted:

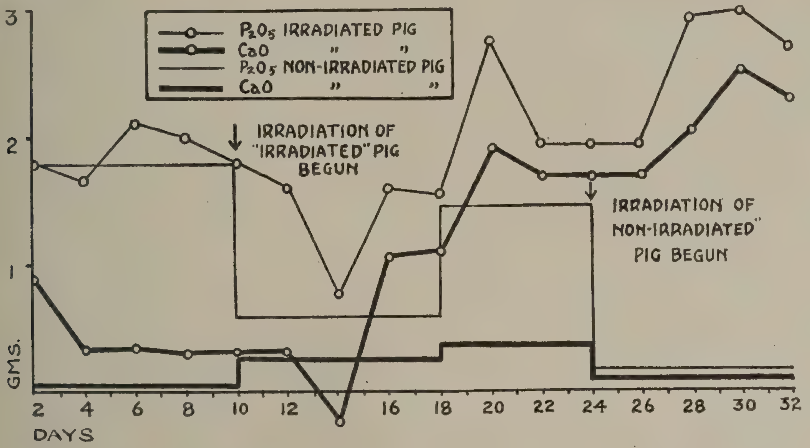

Fig. 1. Retention of calcium and phosphorus in irradiated and non-irradiated pigs.

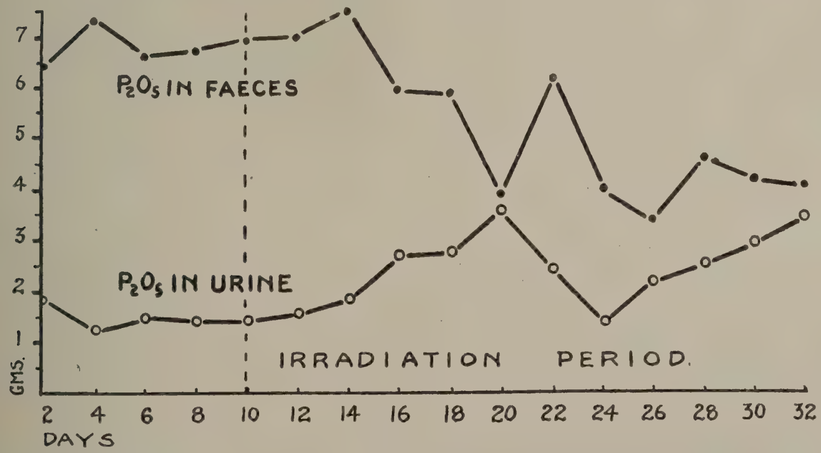

Fig. 2. Showing rise in urinary and fall in faecal excretion of P_2O_5 in irradiated pig.

(1) Both curves show a dip immediately after irradiation was begun, the pig actually reaching a negative calcium balance on the fourth day of irradiation. Thereafter there is a definite and steady rise. The rise is most marked in the case of the calcium retention. The average retention of CaO per diem during the pre-period was ·46 g.; during the irradiation period it rose to an average of 1·45 g. The corresponding figures for P_2O_5 retention were 1·88 g. and 2·07 g. respectively.

(2) The P_2O_5 and CaO balance curves approach each other in the experimental period and run a parallel course.

(3) The ratio of the urinary to the faecal excretion of calcium and phosphorus rises markedly during the experimental period. As regards the calcium, of the total CaO excreted in the pre-period 2·51 % was excreted in the urine; in the experimental period this figure rose to 6·94 %. An average of 18·21 % of the total P_2O_5 was excreted in the urine during the pre-period, and of 32·59 % during the experimental period. Fig. 2 shows the rise in urinary and fall in faecal excretion of P_2O_5 which occurred during the experimental period. It will be seen that, towards the end, the excretion in faeces and in urine was almost equal.

Non-irradiated Pig. After the tenth day of the experiment the non-irradiated pig suffered from gastro-intestinal disturbances, periods of diarrhoea and constipation alternating. As a result of these conditions balances became very irregular and it was impossible to graph 48-hour results. By averaging varying periods, however, it was obvious that the balances were gradually falling. This is not surprising in view of previous results [Husband, Godden and Richards, 1923] in which "pigs kept in metabolic cages and fed on a grain ration showed a progressively decreasing capability of absorbing and retaining CaO and P_2O_5." In the present case the average balances will be seen from Table I. Fig. 1 also shows the marked contrast between the rising retention of the irradiated pig and the falling retention of the non-irradiated pig. In the case of the latter, average periods are graphed: in the former, however, the balance from day to day is set out. The clinical picture corresponded with these findings, for the non-irradiated pig became less active than the irradiated, showed some loss of appetite and, on one occasion at least, vomited its food. In this condition irradiation, when applied for 10 days before the animal was killed, failed to have a beneficial effect; indeed, it possibly accelerated the downward trend. The balances at least became more irregular still. There was a rise in the ratio of urinary to faecal excretion with respect to both CaO and P_2O_5. Thus before irradiation 2·08 % of the CaO excreted appeared in the urine; after irradiation 3·36 % was thus excreted. As regards the P_2O_5, before irradiation 21·3 % was excreted in the urine; after irradiation this proportion rose to 29·8 %.

Table I shows the weight increments for the irradiated and for the non-irradiated pig. The weight gained over six weeks is remarkably small. When the pigs were put in the metabolism cages each weighed 21·0 kg. Forty days later, *i.e.* at the end of the experiment, the non-irradiated pig weighed 22·2 kg., and the irradiated pig 22·4. The latter, however, showed no signs of malnutrition.

Post-mortem Findings. The following are the results of the analyses of the blood and bones. In each case the right radius and ulna and the right tibia and fibula were removed and analysed separately.

	Irradiated Pig 1193		Non-irradiated Pig 1192		Pig 1168		Pig 1112	
	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius
t weight of bones ...	140·99 g.		108·93 g.					
ash in dry fat-ex- racted bone	45·86	43·39	39·77	41·23	43·00	43·92	39·48	39·77
CaO in dry fat-ex- racted bone	24·25	23·35	20·84	22·17	21·28	22·86	20·73	21·02
P_2O_5 in dry fat-ex- racted bone	20·32	18·39	18·12	17·39	18·71	19·51	15·36	16·92
od: CaO	12·9 mg. per	100 cc.	16·3 mg. per	100 cc.				
P_2O_5	138·4 ,,	100 ,,	143·1 ,,	100 ,,				

(Figures for two pigs, 1168 and 1112, from another experiment are placed here for comparison. These pigs showed definite clinical symptoms of rickets and on post-mortem examination there was "marked beading of the costochondral junctions" and "very marked beading" respectively, as also definite histological appearances of rickets.)[1]

In neither animal was any pathological condition found. Neither gross nor histological signs of rickets were observed in the bones. What appeared to be the only abnormality was an enlarged thyroid in the case of the non-irradiated pig.

Experiment 2. *Object.* Having obtained a definite effect of irradiation on the calcium and phosphorus balances when a badly balanced diet was being fed, it was determined to investigate the effects of irradiation, darkness and diffuse light when the animals were on a well-balanced ration.

Animals. Three comparable hog pigs, eleven weeks old were selected from the same litter.

Housing. The general conditions were identical with those in Experiment 1. As regards lighting, there were the following differences between the experimental conditions of the three animals.

(1) Pig 1321 was kept in a darkened metabolism cage and received no irradiation.

(2) Pig 1322 was kept under the same conditions, but had one hour's irradiation daily.

(3) Pig 1323 was kept in a metabolism cage without a canopy. That is, it was in the ordinary diffuse light of the animal room; it received no irradiation.

The pig in the dark and the irradiated pig occupied the cages for 66 days; the irradiation period of the latter was 42 days. The pig in diffuse light occupied the cage for 45 days.

Diet. The diet fed daily consisted of oatmeal, maize-meal, middlings—400 g. each. To this was added 50 cc. of a 10 % solution of NaCl; 20 g. $CaCO_3$, 40 g. blood-meal and 10 cc. olive oil. In the case of the irradiated pig and of the pig kept in the dark, 30 cc. of cod-liver oil was added to their daily ration from the 32nd and the 44th day respectively of the experiment.

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[1] I am indebted to Dr J. P. McGowan for these specimens.

The intake per diem on the above ration was 11·94 g. CaO, 12·77 g. P_2O_5 and 28·83 g. N. The mineral ratios of this diet are roughly as follows:

	Diet	Sow's milk
$CaO : P_2O_5$	12 : 13	10 : 9
$CaO : MgO$	12 : 5	20 : 1

It is thus a better balanced diet than that employed in Experiment 1, particularly with respect to its calcium, phosphorus and magnesium content. NaCl and olive oil were added on account of their beneficial effect as indicated in some experiments at this Institute. The fat-soluble vitamin content of the ration is low. All experimental conditions were similar to those of Experiment 1.

In the case of the irradiated pig the analyses were carried out for 50 days. This was made up of an 8 days pre-period, and 42 days irradiation: during the last 18 days it was receiving in addition 30 cc. of cod-liver oil daily. Analyses of the excreta of the pig kept in darkness were conducted for 50 days, during the last six of which it received daily 30 cc. cod-liver oil. The analysis period of the pig in diffuse light lasted 34 days. Sixty-six days after being first put in the cages the irradiated pig and the pig in darkness were killed. Autopsies were done and analyses of the blood and bones.

Results. Table II (p. 54) shows the balance in the case of the irradiated pig. It will be seen that following irradiation there is a slight dip, but this is later compensated for. Despite oscillations, the balances during the irradiation period show no very definite tendency up or down. Since it was thought that the animal might be on a maximum retention of CaO and P_2O_5, 30 cc. of cod-liver oil was added to the daily diet on the 32nd day. There followed a depression and later an elevation of the balance: the average, however, remained practically the same as in the preceding period. In this connection it must be remembered that the animal had from the beginning of the experiment been receiving olive oil [Husband, Godden and Richards, 1923].

Table II gives also the average balances of the pig kept in darkness. On the whole they show no marked tendency to rise or fall. The cod-liver oil period towards the end is admittedly short, but sufficient to have shown a rise had such been striking. A characteristic feature of the table is the tendency of the P_2O_5 balance to fall below that of CaO. Further the P_2O_5 and CaO curves when graphed in detail show a decided departure from their general parallelism on the 20th and on the 42nd day. On these dates the CaO balance is raised and the P_2O_5 markedly depressed.

The parallelism of the calcium and phosphorus curves in this animal is not so striking as in the irradiated pigs of either Experiment 1 or 2, or as in the "diffuse light" pig of the latter experiment.

It will be noticed from Table II that the balances of the pig in diffuse light start at a slightly lower level than in the other two pigs. In this case, however, the tendency is undoubtedly upwards during the whole metabolic period with the P_2O_5 in general above the CaO.

The following table gives a rough comparative idea of the tendencies of the balances in the three cases. It will be seen that while the intervals represent the pre-period, the period of irradiation alone, and the period of irradiation + cod-liver oil in the irradiated pig these merely correspond to arbitrary periods in the other animals. The table, however, serves the purpose of a broad summary.

Average retention of CaO *and* P_2O_5 *per day.*

	Irradiated pig		Pig in darkness		Pig in diffuse light		
Period	CaO	P_2O_5	CaO	P_2O_5	CaO	P_2O_5	Remarks
First 8 days	5·0	5·2	4·4	4·7	3·7	4·4	Pre-period for irradiated pig
Next 24 days	4·9	5·2	4·8	4·2	4·7	5·0	Light period for irradiated pig
Next 18 days	4·9	5·2	4·9	4·6	—	—	Irradiated pig, light: cod-liver oil. Darkness pig: 6 days cod-liver oil.

As regards the ratio of urinary to faecal excretion of CaO and P_2O_5 there is little to note. It varies within fairly narrow limits and shows no special tendencies.

The weight increments of all three pigs were comparable and show a steady rise (Table II). The irradiated pig put on, on the average, ·34 kg. per day, the pig in darkness ·35 kg. and that in diffuse light ·36 kg. The last, however, had been about 1·5 kg. lighter than the others at the beginning of the experiment.

Post-mortem Findings. The following are the results of the analysis of the blood and bones of the irradiated pig and that kept in the dark. In each case the left ulna and radius were used for analysis.

	Irradiated pig. Ulna and radius	Pig in darkness. Ulna and radius
Wet weight of bones	99·2 g.	101·9 g.
% ash in dry fat-extracted bone	55·65	52·48
% CaO in dry fat-extracted bone	29·57	28·06
% P_2O_5 in dry fat-extracted bone	21·44	19·90
Blood: CaO	9·43 mg. per 100 cc.	9·24 mg. per 100 cc.
P_2O_5	121·2 ,, 100 ,,	98·5 ,, 100 ,,

No abnormal condition was observed in either pig. Sections of the ribs showed normal calcification in each case.

Discussion of Results.

In considering the results of Experiments 1 and 2 the main difference is the marked contrast between the irradiated pig and its control in Experiment 1, and, on the other hand, the comparatively small differences in the second experiment between the irradiated pig, the pig in diffuse light and that kept in darkness. We are thrown back upon the conception that the balance of the diet is of fundamental importance [Orr, 1924]. When this is well adjusted the influence of light would seem to be at a minimum; for all three pigs in Experiment 2 grew normally, remained in good condition and, on the showing of their balance curves, retained adequate and fairly consistent amounts of calcium and phosphorus. On the other hand, in Experiment 1 neither pig grew, and the irradiated pig, which retained more calcium and phosphorus, alone remained in fair condition.

In the first experiment irradiation caused a marked rise in the retention of calcium and phosphorus but it is noteworthy that in each pig there was a slight dip in the balance curves following immediately upon irradiation; in the experimental pig a negative calcium balance was actually reached. Even in the irradiated pig of Experiment 2 there is a suggestion of a depression immediately after irradiation. This may be a specific effect of the light representing a negative phase or at least a period of re-adjustment. On the other hand the depressions of the curves in Experiment 1 were contributed to by an increased faecal excretion, for both pigs after irradiation suffered from diarrhoea for some days. Grant and Gates [1924] report cases of diarrhoea occurring among rabbits after irradiation.

Apart from stimulating the retention of calcium and phosphorus it appears that irradiation has a stabilising effect. Under its influence these minerals are probably retained in an optimum ratio. Both in Experiments 1 and 2 there is a well defined parallelism of the calcium and phosphorus retention curves during irradiation.

The relation of urinary to faecal excretion demands some notice. In the irradiated pig in Experiment 1 one of the most marked results of irradiation was the increase in urinary phosphorus and calcium. It will be seen from Fig. 2 that, while the total excretion was diminishing, the urinary excretion was steadily increasing. The same result, though in lesser degree, was obtained when in turn the non-irradiated pig was irradiated (Table II). There was, however, no such result following upon irradiation in Experiment 2. The increase in urinary excretion is a feature, then, of irradiation of the pig on a badly balanced diet; it is at least not marked when the diet is well balanced. In the treatment of rachitic children a similar result may be observed [Orr, *et al.*, 1923]. This increased excretion *via* the kidney would indicate that there has been on the part of the intestine a greater absorption, a diminished excretion, or both.

In view of recent work [Telfer, 1923] it is probable that the calcium and phosphorus of the faeces consist mainly of unabsorbed residues. If this be so, the effect of the irradiation is to cause an increased absorption by the bowel wall and thus present a greater amount of calcium and phosphorus to the organism. From this reasoning it is obvious that the increase in CaO and P_2O_5 absorbed by the bowel under the influence of light is even more than what is indicated by a graph of the total retention.

The increase in urinary excretion, particularly of phosphorus, also suggests that it is possible as the result of irradiation to induce absorption by the gut at a rate greater than the utilising mechanism can cope with. In the case of the non-irradiated pig in Experiment 1 there was, judging by the criterion of increased urinary excretion, increased absorption but little evidence of utilisation.

It might have been expected that even on a well-balanced diet and a maximum retention of calcium and phosphorus there would have been an increase in the amount of these minerals in the urine following irradiation. Table II indicates that there was no increased absorption with rejection of the superfluous calcium and phosphorus. It may be that not only was the animal retaining a maximum but that in the circumstances a maximum was being absorbed by the wall of the gut. Thus neither irradiation nor cod-liver oil could cause any increase in absorption.

The failure of growth in the irradiated pig in Experiment 1 is striking. By many it is assumed that growth is essential to rickets [Parks, 1923] and it seems at first sight an attractive theory that irradiation may in part exercise its anti-rachitic effect through inhibiting undue general growth; that failure to grow is really a protective mechanism. There are, however, too many results showing growth under the stimulus of light to make this idea tenable. At the other extreme there are workers who, as a result of experiment, hold that the "growth factor" and the "anti-rachitic factor" are identical [Steenbock and Nelson, 1923]. In the present instance of the irradiated pig in Experiment 1 it is obvious that light may stimulate the retention of calcium and phosphorus without causing any concomitant increase in general body growth. Webster and Hill [1924] have recently pointed out an interesting example which, in their opinion, suggests that "the radiant energy necessary to promote growth appears to be other than that required to cure rickets." In the case of a rat which on irradiation showed no increase in growth over the controls, the P_2O_5 content of the carcass was nevertheless appreciably higher.

The analyses of the bones as detailed above reveals a difference in the direction one would have been led to expect. Thus, averaging the two sets of bones in Experiment 1, it is found that those of the irradiated pig contain 4·6 % more CaO and 3·2 % more P_2O_5 than those of the pig in darkness. As regards the corresponding pigs in Experiment 2 the figures are respectively 1·15 % and 1·14 %. That a terminal analysis of bones, however—unless it be the whole skeleton—gives only a rough indication of the amounts of CaO and P_2O_5 absorbed and deposited, is seen from the fact that had the ulna and radius alone been compared in corresponding pigs no marked difference would have been observable.

It will be noticed that in Experiment 2 the irradiated pig has a slightly higher blood calcium and a markedly higher blood phosphorus. The phosphorus retention in this pig had been greater than in the pig kept in darkness. But in Experiment 1 the control pig has a higher blood content of both minerals. It must be remembered, however, that this control had been irradiated for the previous 10 days and that while it was not retaining and depositing calcium and phosphorus it was absorbing considerable quantities as is shown by the urinary excretion of CaO and P_2O_5. It is not surprising that the blood calcium and phosphorus are high after irradiation, particularly in an animal which is not depositing these minerals. It is doubtful, however, if much can be deduced from the figures for the blood analysis. There are possibly in pigs marked individual differences in the CaO and P_2O_5 content of the blood. Had it been possible the variation under light treatment would have been followed out in each individual pig but this was found incompatible with the success of the metabolic experiment since it was feared that the disturbance occasioned the pig by taking a blood sample might upset the balances.

SUMMARY.

(1) In a pig on a diet badly balanced particularly with respect to phosphorus, calcium and magnesium (P_2O_5 : CaO : MgO—3 : 1 : 1) 1 hour's irradiation daily for 24 days with a carbon arc lamp definitely increased the calcium and phosphorus retention as compared with a control animal kept in the dark. The balance curves for these minerals tended to approach each other in the light period and run a parallel course.

(2) Following upon irradiation the calcium and phosphorus in the urine rose, both actually and relatively to their excretion in the faeces, suggesting an increased absorption from the gut or a decreased excretion.

(3) The above results took place in the irradiated pig without any concomitant increase in body weight. This would indicate that a stimulus to calcium and phosphorus retention does not necessarily involve a stimulus to growth in general.

(4) In pigs on a satisfactory diet, well balanced particularly with respect to calcium and phosphorus (P_2O_5 : CaO—1 : 1), darkness, diffuse light (through glass) and irradiation of 1 hour per day for 52 days caused little difference in the calcium and phosphorus retention, though such difference as there was, was in favour of the irradiated pig.

(5) In the irradiated pig on a well-balanced diet no tendency was observed for the urinary calcium and phosphorus to increase after irradiation.

(6) Analyses of the bones were in general agreement with the findings of the metabolic analyses.

I wish to express my indebtedness to Dr J. B. Orr, Director of the Rowett Institute, on whose suggestion the above work was undertaken; also to Dr H. E. Magee and Mr G. A. Reay for valuable assistance in the analytical work.

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IX. THE BACTERICIDAL ACTION OF THE BLOOD IN CERTAIN DIETARY DEFICIENCIES.

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In the present paper an attempt has been made to determine the effects of diets deficient in vitamins and in certain other essential factors on the bactericidal power of whole blood.

Zilva [1919] has shown that the power to form agglutinins, complement and immune body is not decreased in the rat as the result of diets lacking in vitamins, while Werkman [1923] has found that both in the rat and rabbit there is no inability to form agglutinins, precipitins, haemolysins and bacteriolysins on diets deficient in vitamins A and B. Findlay and Mackenzie [1922], confirmed by Werkman [1923], could find no defect in the opsonic power of the blood. Smith and Wason [1923] however stated that the bacteriotropic and bactericidal action of the serum on *B. typhosus* was reduced in rats fed on diets lacking vitamins A and B. In the case of guinea-pigs fed on a diet deficient in vitamin C, Findlay [1923] noted a diminished leucocytic response to bacterial infection, while in the case of pigeons lacking vitamin B the peritoneal exudate had less bactericidal power than that of control birds.

The present investigations have been carried out on the bactericidal power of the whole blood for *Staphylococcus aureus* in normal rats and in rats subjected to the following procedures:

A. Fed on a diet lacking vitamin A and the antirachitic factor.

B. Fed on a diet lacking vitamin A and the antirachitic factor and exposed to ultra-violet light.

C. Fed on a diet lacking the antirachitic factor and phosphorus.

D. Fed on a diet lacking vitamin B.

The rats used in these experiments were all bred from laboratory stock. As preliminary experiments had shown that the employment of anaesthetics in killing the animals caused variations in the bactericidal power of the blood it was decided to kill the rats by a blow on the back of the head; the thorax was then opened and the blood was removed from the heart with aseptic precautions. The rats used as controls were fed on laboratory diet with the addition of cod-liver oil and marmite. All the animal experiments were carried out by one of us (G. M. F.). The observations on the bactericidal power of the blood were made by the slide cell method of Wright, Colebrook and Storer [1923].

A. RATS FED ON A DIET DEFICIENT IN VITAMIN A AND THE ANTIRACHITIC FACTOR.

The rats used in this experiment were placed on the following diet:

Caseinogen	14 g.
Starch	77
Marmite	5

Inorganic salt mixture:

NaCl	0·32 g.	$CaH_4(PO_4)_2 . 2H_2O$	1·02 g.
$MgSO_4$	0·56	Ca lactate ...	2·46
$NaH_2PO_4 . 2H_2O$	0·66	Fe citrate ...	0·22
K_2HPO_4 ...	1·76		

Though lacking both vitamin A and the antirachitic factor, animals placed on this diet do not develop rickets since the proportions of calcium and phosphorus are correct.

In the first experiment the rats were allowed to continue on the diet until they began to show symptoms due to a deficiency of vitamin A. The body temperature at the time of death and the bactericidal power of the blood are shown in Table I.

It will be seen that the body temperature of the normal rat varies from about 37·0° to 38·0°, while the percentage of staphylococci killed by the blood is as a rule about 70. There is thus a definite fall in the bactericidal power of the blood in association with a deficiency of vitamin A and the antirachitic factor, but only after the onset of keratomalacia or broncho-pneumonia.

In the second experiment young rats were placed on the diet deficient in vitamin A and the antirachitic factor; they were then killed after varying periods on the diet, a random selection being made. Here again, as shown in Table II, there is no fall of bactericidal power until after the onset of keratomalacia.

B. RATS FED ON A DIET DEFICIENT IN VITAMIN A AND THE ANTIRACHITIC FACTOR, BUT EXPOSED TO ULTRA-VIOLET LIGHT.

The rats were placed on the same diet as in the previous experiments but were irradiated for 20 minutes each day with a mercury vapour lamp at a distance of 8 inches.

The experiments of Colebrook, Eidenow and Hill [1924] have shown that when a healthy animal is exposed to ultra-violet light there results an increase in the bactericidal power of the blood: this increase lasts for at least 2 hours after the exposure but tends to disappear 4 hours after exposure. The rats were killed after varying periods on the diet and, except in two cases, from 6 to 24 hours after exposure to the ultra-violet light. It will be seen from Table II that the bactericidal power of the blood of the animals exposed to light did not fall in the same way as in those kept in diffused daylight; this must be correlated with the fact that the animals exposed to ultra-violet light did not develop keratomalacia or other intercurrent infections so rapidly as the non-exposed rats. The growth curves also of the irradiated rats were better than those of the non-exposed rats, a confirmation of the findings of Hume [1922] and Goldblatt and Soames [1922].

Table I. *The Bactericidal Power of the Blood in Control Rats and in Rats on a Diet deficient both in Vitamin A and the Antirachitic Factor.*

No. of rat	Body temperature at death	Percentage of staphylococci killed	Remarks
		Vitamin-rich diet.	
1	37·7	72	—
2	37·1	65	—
3	37·4	67	—
4	37·3	74	—
5	37·2	73	—
6	37·6	76	—
7	37·5	68	—
Average	37·4	71	—
		– Vitamin A and – Antirachitic Factor.	
8	37·2	67	–Keratomalacia
9	37·2	46	+ ,,
10	37·7	60	– ,,
11	37·2	71	– ,,
12	36·9	50	– ,, +Broncho-pneumonia
13	37·6	65	+Keratomalacia
14	36·5	43	+ ,,
15	37·1	38	+ ,,
16	37·2	53	+ ,,
17	37·1	45	+ ,,
18	37·2	55	+ ,,
19	37·5	57	– ,, +Broncho-pneumonia
Average	37·2	54	—
		– Vitamin A and – Antirachitic factor. Single exposure to Ultra-violet Light 1 hour before death.	
20	37·2	48	+Keratomalacia
21	35·0	47	+ ,,
22	37·1	45	+ ,,
23	37·2	54	+ ,,
Average	36·6	48·5	—

Four rats suffering from keratomalacia were exposed to ultra-violet light for 30 minutes and the bactericidal action of the blood tested 1 hour later. As shown in Table I the bactericidal action of the blood in these animals was still low after irradiation. This is in agreement with the observations of Colebrook [1924], who found that patients with a low bactericidal power of the blood do not show any increased bactericidal action of the blood after exposure to ultra-violet light.

Table II. *The Bactericidal Power of the Blood of Rats on a Diet deficient both in Vitamin A and the Antirachitic Factor and killed after varying periods on the Diet.*

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed	Remarks
		Vitamin-rich diet.		
24	—	37·4	78	—
25	—	37·6	79	—
26	—	37·8	76	—
27	—	37·2	76	—
28	—	37·1	67	—
29	—	38·0	70	—
30	—	37·4	75	—
Average	—	37·5	74	—
		– Vitamin A and – Antirachitic factor.		
31	7	37·6	75	– Keratomalacia
32	7	37·9	78	– ,,
33	7	37·7	77	– ,,
34	33	36·7	43	+ ,,
35	33	35·4	38	+ ,,
36	54	36·7	47	+ ,,
37	54	36·8	45	+ ,,
Average		37·0	58	—
		– Vitamin A and – Antirachitic factor. Daily exposure to Ultra-violet Light.		
38	7	37·2	78	– Keratomalacia
39	7	37·4	77	– ,,
40	7	37·6	76	– ,,
41	26	37·3	90	– ,, } killed 1 hour after exposure to ultra-violet light
42	26	37·0	92	– ,,
43	33	37·8	75	– ,,
44	33	38·0	71	– ,,
45	54	37·7	69	– ,,
46	54	38·4	72	– ,,
47	78	38·0	72	– ,,
48	78	38·0	69	– ,,
49	92	36·9	54	+ ,,
Average	—	37·7	74	—

C. RATS FED ON A DIET LACKING THE ANTIRACHITIC FACTOR AND PHOSPHORUS.

When young rats are placed on the following diet [Pappenheimer, McCann and Zucker 1922]:

White flour	80·9 g.
Egg albumin	10·0
Butter fat	5·0

Salt mixture:

KCl	0·850 g.	Ca lactate	2·000 g.
Na_2CO_3	0·850	Fe citrate	0·100
$MgCO_3$	0·286	KI	0·0002

they invariably develop rickets. The diet is adequate for full growth since it contains both vitamins A and B; it can be rendered antirachitic by the addition of cod-liver oil or phosphates or by exposing the animals to ultraviolet light. The rats in this experiment were allowed to remain on the diet for 3 weeks before testing the bactericidal power of the blood. All the rats had well-developed rickets. Reference to Table III shows that in two cases the bactericidal power of the blood was normal while in four cases it was greatly reduced.

Table III. *The Bactericidal Power of the Blood of Rats on a Diet deficient in the Antirachitic Factor and Phosphorus.*

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed
	Vitamin-rich diet.		
50	—	37·4	68
51	—	37·2	74
52	—	37·6	76
53	—	37·8	67
Average	—	37·5	71
	Rickets Rats.		
54	21	36·9	30
55	21	37·2	20
56	21	37·2	48
57	21	37·5	74
58	21	36·8	70
59	21	36·8	30
Average	—	37·1	45

Table IV. *The Bactericidal Power of the Blood of Rats on a Diet deficient in Vitamin* B.

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed
	Vitamin-rich diet.		
60	—	37·7	75
61	—	37·1	65
62	—	37·1	67
63	—	37·3	72
64	—	37·2	73
Average	—	37·3	70
	— *Vitamin* B.		
65	25	35·6	42
66	25	35·4	50
67	32	35·1	38
68	32	35·1	33
69	35	<35·0	38
70	35	<35·0	39
71	39	35·2	50
72	39	<35·0	50
73	41	36·2	51
Average	34	35·3	43

D. RATS FED ON A DIET LACKING VITAMIN B.

In this experiment the rats were fed on the following diet:

Caseinogen	14 g.
Starch	77
Cod-liver oil	5

Inorganic salt mixture:

NaCl	0·32 g.	$CaH_4(PO_4)_2 . 2H_2O$	1·02 g.
$MgSO_4$	0·56	Ca lactate ...	2·46
$NaH_2PO_4 . 2H_2O$	0·66	Fe citrate ...	0·22
K_2HPO_4 ...	1·76		

The length of time on the diet, the body temperature and the bactericidal action of the blood are shown in Table IV. A fall in the bactericidal action of the blood occurs as the result of a diet deficient in vitamin B, apart from the occurrence of any bacterial infection, though the lowering of the bactericidal power in this type of deficiency is always associated with a fall in body temperature. In the case of animals fed on diets deficient in vitamin B, this decline in body temperature takes place some days before death.

DISCUSSION.

The question naturally arises as to whether the bactericidal power of whole blood is due to the action of the leucocytes or the serum. Smith and Wason [1923], as previously indicated, found that the bactericidal power of the serum against *B. typhosus* was decreased as the result of diets deficient in vitamins A and B. It is doubtful whether any great significance can be attached to their results, as the bactericidal action on *B. typhosus* of the serum of normal rats is known to vary very greatly. The blood of wild and black rats as a rule has no bactericidal action on *B. typhosus*, while that of white rats has a slight bactericidal action.

The serum of the rat has little or no bactericidal action on *Staphylococcus aureus*.

The work of Wright, Colebrook and Storer [1923], on the other hand, has proved that the bactericidal power of whole blood against *Staphylococcus* is largely due to the polymorphonuclear leucocytes.

Any factor which tends either to reduce the numbers of the polymorphonuclear leucocytes or to impair their functional activity might therefore decrease the bactericidal power of the blood. It is thus of interest to determine whether there is any reduction in the number of the polymorphonuclear leucocytes in the blood in association with vitamin deficiencies.

Total and differential white cell counts were made in 10 normal rats and in 10 rats with keratomalacia as the result of a diet lacking vitamin A. The following figures were obtained:

Control rats:	9450 leucocytes per mm.³	Polymorphs 35 %
Vitamin A rats:	8170 ,, ,, ,,	,, 48 ,,

There is thus little difference in the total leucocyte counts, while in the case of the vitamin A deficient rats there is a polymorphonuclear leucocytosis, probably as a result of the bacterial infection. Cramer, Mottram and Drew [1921] have obtained similar figures. When rats are fed on diets deficient in vitamin B, there is a fall in the total leucocyte count, but this fall only affects the lymphocytes, the polymorphs, as shown by Cramer, Mottram and Drew [1921] exhibiting little or no decrease in number. It is obvious therefore that the fall in bactericidal power of the blood in vitamin A and B deficiencies cannot be due to a decrease in the number of polymorphonuclear leucocytes.

There is, however, a possibility that the killing power of all the polymorphonuclear leucocytes in the blood is not equal. If young and aged polymorphonuclear leucocytes are of less vigour than adult cells, then the bactericidal power of the blood should tend to decrease whenever there is an excessive proportion of very young or very old polymorphs in the blood stream, unaccompanied by a definite leucocytosis. An excessive number of aged polymorphonuclear leucocytes for instance would survive in the blood stream in a condition where there is diminished leucoblastic activity of the bone marrow unassociated with bacterial infection. Such a condition is found in animals fed on diets deficient in vitamin B, where the bone marrow, as shown by Shipley, McCollum and Simmonds [1921], exhibits numerous small haemorrhages. In addition Happ [1922] has found that in rats suffering from a deficiency of vitamin B there is a very high proportion of aged or moribund polymorphs in the blood stream.

CONCLUSIONS.

1. The blood of normal rats, tested against *Staphylococcus aureus*, shows a killing power of about 70 %.

2. Rats fed on a diet deficient in vitamin A and the antirachitic factor show a reduction in the bactericidal power of the blood only after the onset of keratomalacia or some other infection.

3. In rats fed on a diet deficient in vitamin A and the antirachitic factor, but exposed to ultra-violet light, the onset of bacterial infection and the reduction in the bactericidal power of the blood are delayed.

4. Rats suffering from keratomalacia as the result of a diet deficient in vitamin A continue to show a low bactericidal power of the blood after exposure to ultra-violet light.

5. Rats fed on a diet lacking phosphorus and the antirachitic factor show, in most cases, a reduction of the bactericidal power of the blood.

6. Rats fed on a diet lacking vitamin B show a reduction in the bactericidal power of the blood unassociated with bacterial infection but possibly correlated with the fall in body temperature.

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X. THE DETERMINATION OF CYANATES IN BLOOD.

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The occurrence of cyanic acid during the oxidative deamination of glycine, alanine and sarcosine has been quoted recently in support of the hypothesis that this acid is an important intermediate product in the formation of urea in the animal body [Fearon and Montgomery, 1924]. Additional support was claimed from the work of Nicloux and Welter [1922], as reviewed by Riggs in *Chemical Abstracts* [1923].

At the time the original paper was not available, but a subsequent study of the work revealed the unexpected fact that Nicloux and Welter do not claim to have obtained any positive evidence for the occurrence of cyanic acid in the blood of the dog. Their data, however, are also capable of the interpretation given by Riggs. "Ten parts of plasma of dog blood mixed with 1 part of satd. NH_4Cl soln. and incubated 36 hrs at 35° showed an increase of about 5 % in the urea content over that of the initial plasma. By adding 0·1 mg. KCNO to 1 cc. of plasma-NH_4Cl mixture, the increase of urea in 36 hrs was 21 %. Cyanates therefore occur in blood" [Riggs, 1923].

With the object of removing this ambiguity, the blood of the rabbit and the cat was examined for cyanate by a method similar to that of Nicloux and Welter with slight modifications.

In several animals a positive result was obtained. For the rabbit the cyanate value was found to be 0·7 mg. per 100 cc. plasma; and for the cat a value of 1·0 mg. per 100 cc. plasma was obtained, expressed as HOCN.

THE METHOD OF DETERMINING CYANATE IN BLOOD.

This can most readily be accomplished by converting the cyanate into urea by incubation with a suitable ammonium salt solution in accordance with the equation:

$$NaOCN + NH_4Cl = CON_2H_4 + NaCl.$$

This change is not quantitative as some of the cyanate undergoes hydrolysis to ammonia and carbon dioxide, depending on the time and temperature of the incubation, and the concentration of the added ammonium salt. Nicloux and Welter found that a 0·1 % solution of KCNO, incubated at 35° with a saturated solution of NH_4Cl, gave 36 % of the possible urea in 12 hours, and 77 % in 48 hours.

Recovery of cyanate as urea. 20 cc. of a fresh KOCN solution (0·101 %) were incubated with 0·2 g. $(NH_4)_2SO_4$ at 40° for 24 hours. The urea recovered represented 77·1 % of the total cyanate added.

For the determination of the urea the xanthydrol method was applied as in the previous paper [Fearon and Montgomery, 1924]. The more delicate urease methods of estimating urea are not so suitable, since Fearon [1923] and Mack and Villars [1923] have shown the formation of cyanic acid during the decomposition of urea by urease; consequently confusion might arise between any cyanic acid present in the plasma and cyanic acid formed by the influence of the enzyme.

Details of method. 30 to 40 cc. of plasma were obtained by centrifugalisation of the fresh blood and divided into two equal samples, A and B. A, the control, was treated with toluene and incubated in a sterile tube for 24 hours at 43°. 0·2 g. of ammonium sulphate was added to B, which was incubated for the same time under the same conditions. An equal volume of concentrated Tanret's reagent[1] was then added to each tube, and the contents filtered. The filtrates were treated with equal volumes of glacial acetic acid, and 1 cc. of a 10 % solution of xanthydrol in methyl alcohol.

At the end of 12 hours the precipitates of dixanthylurea were filtered off, washed with 3 cc. absolute alcohol saturated with dixanthylurea, dried and weighed, and the weight of the urea present determined by dividing the weight of the dixanthylurea by 7.

The solubility of the dixanthylurea in the acetic acid mixture under the conditions of the experiment was determined and found to be 1·6 mg. per 100 cc.

Table I. *Recovery of urea added to 5 cc. of blood plasma.*

Exp. 1.	Initial urea content of plasma	2·23 mg. per 5 cc.
	Final urea content of plasma	3·74 ,, ,, 5 ,,
	Estimated amount of urea added ...	1·51 ,, ,, 5 ,,
	Actual amount of urea added	1·56 ,, ,, 5 ,,
Exp. 2.	Initial urea content of plasma	1·53 mg. per 5 cc.
	Final urea content of plasma	3·68 ,, ,, 5 ,,
	Estimated amount of urea added ...	2·15 ,, ,, 5 ,,
	Actual amount of urea added	2·20 ,, ,, 5 ,,

Table II. *Cyanic acid concentration of rabbit plasma.*

Rabbit. Three hours after meal of 130 cc. of milk. Urea determined in 10 cc. of plasma.

Urea content of plasma A	55·3 mg. per 100 cc.
,, ,, ,, ,, B	56·4 ,, ,, 100 ,,

Increase represents 0·78 mg. cyanic acid per 100 cc.

Table III. *Cyanic acid content of cat plasma.*

Urea determinations in 12·5 cc. of plasma before and after incubation with ammonium sulphate.

Cat I.	Urea content of plasma A	63·3 mg. in 100 cc.
	,, ,, ,, ,, B	64·7 ,, ,, 100 ,,
	Urea increase represents 1·04 mg. cyanic acid in 100 cc.	
Cat II.	Urea content of plasma A	61·3 mg. in 100 cc.
	,, ,, ,, ,, B	63·17 ,, ,, 100 ,,
	Urea increase represents 1·05 mg. cyanic acid in 100 cc.	

[1] Tanret's reagent: mercuric chloride (2·71 g.), potassium iodide (7·2 g.), in 66·6 cc. of glacial acetic acid, made up to 100 cc. with distilled water.

By means of control experiments the limits of error of the method were found to lie within ± 0·2 mg. per 100 cc.

Confirmatory test for cyanate in blood plasma. To determine if the increase in urea due to incubation with the ammonium salt arose from pre-existing cyanate or from some other source, such as the hydrolysis of some unidentified ureide, a determination was carried out on rabbit blood plasma after acidification by the addition of 3 cc. $N/10$ H_2SO_4 to 15 cc. plasma. This would destroy any cyanate present by converting it into free cyanic acid which is rapidly hydrolysed in acid solution. The urea content of this plasma is shown in the following table:

Table IV. *Urea content of acidified plasma after incubation for 24 hours.*

Urea content of plasma A	56·6 mg. per 100 cc.
,, ,, ,, ,, B	56·7 ,, ,, 100 ,,

This confirms the belief that the increase in plasma urea after incubation with ammonium salts is due to the presence of cyanate, since previous acidification of the plasma checks the formation of urea.

SIGNIFICANCE OF CYANIC ACID IN THE BLOOD.

Since it is so closely related to urea, the occurrence of cyanic acid in the plasma suggests that it is either a precursor or a product of the urea formed in the organism. It may represent amino-nitrogen on its way to be converted into urea, or it may result from the enzyme dissociation of urea. The evidence available is strongly in support of the former view. There is at present no ground for the belief that the enzyme urease is present in the tissues of the human body, and considerable indirect evidence that it does not occur. Perfusion of surviving dog's liver with dog's blood, saline, and urea (up to 4 g. per litre) showed no increase in ammonia; consequently, Wakeman and Dakin [1911] concluded that the formation of urea appeared to be an irreversible reaction. Luck [1924] has recently demonstrated the presence of urease in the gastric mucosa of the Carnivora and Ruminantia, but this may be due to ferments taken in with the food or arising from micro-organisms in the food. The possibility of extraneous infection must always be borne in mind in working with urea as Werner [1918] has shown; the dissociation of urea in aqueous solution at low temperature is due, probably entirely, to infection. The number of micro-organisms capable of decomposing urea is very large.

The occurrence of a variable amount of cyanate in plasma will also explain the varying values for blood ammonia obtained by different observers. Solutions of cyanate in low concentration may be completely hydrolysed to ammonia and carbon dioxide by prolonged aspiration at room temperature; and the earlier and more drastic methods for estimating blood ammonia no doubt owe their high values to the unrecognised presence of cyanate.

CONCLUSIONS.

1. Cyanic acid is present in the blood plasma of the cat to the extent of 1·04 mg. %, and in the blood plasma of the rabbit to the extent of 0·78 mg. %, some hours after a protein meal.

2. Its presence is considered to be of significance in the formation of urea in the animal body.

3. The varying results found in the determination of the blood ammonia may be due to the presence of cyanic acid.

I wish to acknowledge my indebtedness to Prof. H. Pringle of this department for facilities afforded in this research.

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XI. A POSSIBLE CORRELATION BETWEEN DIETARY PROTEIN AND LOSS OF FUR IN YOUNG GROWING RATS.

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The importance of protein in the diet has been emphasised by numerous workers. It is impossible here to give even a brief survey of the literature on this subject, but reference might well be made to the interesting and critical paper of Mitchell [1924], who takes a broad view of protein metabolism and gives a considerable number of references.

Loss of hair as a result of a special diet has been noted by several observers. McCollum, Simmonds and Parsons [1918] comment on this phenomenon in rats when the animals were fed on dried potato and butter fat, supplemented by the inorganic elements sodium, chlorine and calcium, in which these observers state that the potato is deficient.

Boas [1924, 1] also reports a baldness in rats fed on a commercial preparation of egg-white as the sole source of protein. Her animals, at the same time, ceased to gain weight; they remained stationary for a week or so and then lost weight. With alterations in the diet, the animals recovered, gained weight again, and new hair appeared in less than a week. Thus the loss of fur was obviously dietetic in origin. In a more recent paper Boas[1] states that no cessation of growth or loss of fur occurs when the rats received the white from fresh English eggs as the sole source of protein [1924, 2].

A loss of hair in rats was commented on by the author in a previous paper [1924]. The animals were fed on a diet of white bread, butter and salt mixture. A loss of hair was noticed when the young rats were about 6 weeks old, *i.e.* after about 3 weeks on the special diet. Eventually the rats' hair grew again *on the same diet*, although not so rapidly as when the diet was altered, *e.g.* when caseinogen was added. This rapid improvement on the addition of caseinogen is quite in agreement with the findings of Boas [1924, 1].

A diet of bread, butter and salt mixture might be regarded as poor from the point of view of protein, and although the rats showed no signs of lack of vitamin B during the $7\frac{1}{2}$ months of the experiment, it might be suggested that the vitamin B content was at a minimum. Thus two possible dietetic factors might be responsible for the loss of fur in the young rats. In the same communication [1924] it was shown that rats fed on a similar diet, with the addition of wheat germ extract, lost their fur in the same manner, so that the addition of more vitamin B to the diet was ineffective. This has been confirmed again by using a 3 % extract of vitamin B made from marmite, instead of wheat germ extract, when the loss of fur occurred just as before. This is also proved by the experiments of Boas, for her rats which showed loss of fur were receiving appreciable quantities of marmite in their diet. It seemed, therefore, highly probable that the deficiency must be looked for in either the quality or quantity (or both) of the protein. The experiments described in this paper were started in the hope of throwing some light on this problem.

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[1] I am indebted to Boas for allowing me to read this paper in proof form.

Experimental.

1. *Animals.* The rats used in this research were piebald animals bred in the laboratory [Hartwell, Mottram and Mottram, 1923]. Twelve rats were used for each experiment, 6 ♀ and 6 ♂, and were kept in a large cage together. They were weighed once a day for the first few weeks and afterwards once a week.

2. *Foods.* The animals were given food *ad lib.*; all constituents were well mixed so that the rats could not pick out any one substance. The food was given as a moist paste. In hot weather the rats needed extra water to drink, but in cold weather they drank none.

The diets given were:

1. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. 3 % extract containing vitamin B [Hartwell, 1922, 2].
2. 30 g. brown bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water.
3. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water, 1·5 g. food casein [Hartwell, 1922, 1].
4. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water, 1·5 g. gluten[1] (80·62 % protein).
5. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 84 cc. water, 1·5 g. gelatin (the gelatin was soaked overnight in 18 cc. of cold water).
6. 100 g. oatmeal (medium), 500 cc. water, 2·88 g. salt mixture, 14 g. butter. The oatmeal was cooked for $1\frac{1}{2}$ hours in 400 cc. water, then 100 cc. more water were added together with the butter and salt mixture, and the whole well mixed.
7. 100 g. boiled potato, 25 cc. water, 1 g. salt mixture, 5 g. butter.

Results.

The composite growth curves of the various animals are shown in the figure. The curves of the male animals only are given, the reason being that the does became pregnant at different dates, so that their curves are not strictly comparable one with the other. Growth is only given for 14 weeks, although some of the experiments lasted for a year, and some are still in progress. That there is no deficiency in vitamin B in any of these diets is proved by the continuous, though in some experiments slow, growth for this period. The work is being continued with the view to ascertaining the effect of these various diets on gestation, and results of this will be given in a later paper.

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[1] I am indebted to Callard & Co. for supplying me with an analysis of the gluten used in this work.

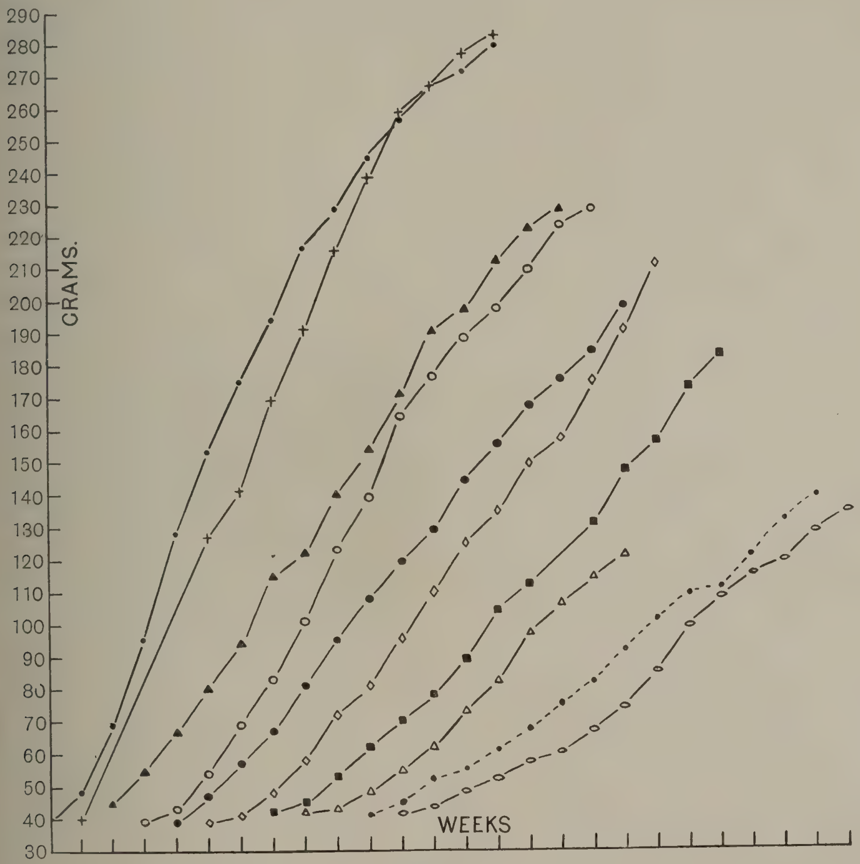

Fig. 1.

• ——— • Control animals. Good mixed diet (kitchen scraps, together with bread and milk).
+ ——— + Diet of white bread, butter, salt mixture and food casein.
▲ ——— ▲ Diet of oatmeal (medium), butter and salt mixture.
○ ——— ○ Diet of white bread, butter, salt mixture and gelatin.
● ——— ● Diet of white bread, butter, salt mixture and gluten.
◇ ——— ◇ Diet of brown bread, butter and salt mixture.
■ ——— ■ Diet of white bread, butter, salt mixture and extract containing vitamin B made from marmite.
△ ——— △ Diet of white bread, butter, salt mixture and wheat germ extract. (Taken from Hartwell [1924].)
• - - - - • Diet of white bread, butter and salt mixture. (Taken from Hartwell [1924].)
⬭ ——— ⬭ Diet of boiled potato, butter, and salt mixture.

From examination of the curves, it is obvious that all the animals grew, but at very different rates. The fur came off when the animals were fed on the potato and white bread diets, even when wheat germ extract or extract made from marmite containing vitamin B was added to the bread. With the potato diet, the fur grew again, but *very* slowly, and the new coat was poor. Old potatoes were used in this experiment but it is being repeated with new ones to see if there is any difference. With the bread diets, the fur also grew again, and when grown was in good condition. The growth of fur was more rapid than with the potato-fed rats, but not nearly so quick as when caseinogen was added to the bread diet.

COMMENTS.

From these experiments it appears that the slow rate of growth is correlated with the falling of the animals' hair, but this may be mere coincidence. If the loss of fur in Boas' [1924, 1] experiments is due to the same dietetic error the slow rate of growth does not necessarily coincide with the falling hair, because her animals were growing more rapidly than mine.

It is interesting that the falling of the hair could be entirely prevented by adding more gluten to the bread diet, hence gluten in itself cannot be such a poor protein as is frequently claimed. Also the addition of extra gluten causes a marked improvement in the rate of growth. Gelatin and caseinogen also prevented the fur from coming out, the substitution of brown instead of white bread was similarly effective. From this latter fact alone, the casual observer would remark that the improvement must be due to the greater vitamin B content of brown bread, but that this is an incorrect assumption has been proved by adding wheat germ extract or extract containing vitamin B made from marmite to the white bread diet, when no improvement was obtained. Brown bread actually contains less protein than white bread [Plimmer, 1921], when calculated as dry constituents, but the difference is so slight as to be practically negligible. It is probable that the germ protein is different in composition from the endosperm protein, and although the germ is only a small part of the whole grain, its protein percentage is high [Osborne and Mendel, 1919; "crude protein" 40·25 %]. The author has not been able to find any analyses of the germ protein and therefore this explanation is put forward somewhat tentatively. It is, however, conceivable that some amino acid is absent from the endosperm protein, though present in the germ protein, so that the latter is highly effective in supplementing the former.

Boas [1924, 1] found that 5 g. of spinach per day per rat effected a cure, and although this may appear to be a small amount considering the percentage of water in green vegetables, yet the biological value of spinach protein is high [Lusk, 1917]. It is interesting to note that oatmeal protein is effective in promoting quite good growth, though not up to the control rate.

From the experiments described in this paper, it is justifiable to conclude that the quality or quantity (or both) of the dietary protein is a factor influencing the loss of hair in young growing rats. It is quite possible that a similar condition might be brought about by other dietary deficiencies.

CONCLUSIONS.

1. The loss of fur in a growing rat is closely correlated with the quality or quantity (or both) of the dietary protein.

2. This loss of fur can be remedied by adding more or better protein to the animals' diet.

3. A loss of fur was obtained when potato or white bread was used as sole source of protein. The addition of gluten, gelatin, or caseinogen, to the bread diet completely prevented the loss of fur. Brown bread or oatmeal as sole source of protein resulted in no loss of hair.

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XII. THE OSMOTIC PRESSURE OF HAEMOGLOBIN.

AN EXPLANATION OF THE EFFECT OF ACETIC ACID, BASED ON DONNAN'S THEORY OF MEMBRANE EQUILIBRIUM.

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In a recent publication [Wilson, 1923] on "The Effect of Carbon Dioxide and Acetic Acid on the Osmotic Pressure of Haemoglobin," it was shown that "the osmotic pressure of solutions of purified haemoglobin is three or four times as great when dialysed against acetic acid or water saturated with CO_2 (both about p_H 4) as against water alone." Reasons were given for believing that "this is due to the formation of some sort of ionising salt of haemoglobin, either one which ionises into several protein ions, or into protein and acetate or protein and bicarbonate ions" (p. 70). It was pointed out that a study of the hydrogen-ion concentration of the haemoglobin solution inside the osmometer and the fluid outside the osmometer should determine which kind of dissociation had taken place. If the ionisation were into protein ions only, the Donnan equilibrium [1911] should not come into play and the hydrogen-ion concentration of the inner and outer solutions in the osmometer should be the same when equilibrium was reached. If a haemoglobin acetate were formed which dissociated into haemoglobin and acetate ions, then the Donnan equilibrium would, presumably, occur and the inner solution would be less acid than the outer. The latter hypothesis is substantiated by the experiments to be reported in this paper. The conclusion is, therefore, drawn that the increased osmotic pressure observed is due to the formation of haemoglobin acetate. The resulting equilibrium relations have been quantitatively considered.

EXPERIMENTAL.

The haemoglobin[1] was several times recrystallised according to the method of Dudley and Evans [1921], which depends on the fact that reduced haemoglobin is much more soluble than oxyhaemoglobin. The osmometers consisted of collodion sacs attached to mercury manometers. The method of preparing the purified haemoglobin and the description of the osmometers are fully given in the previous communication [Wilson, 1923]. In the present experiments larger osmometers (holding about 15 cc. haemoglobin) were used in order to have enough haemoglobin solution for duplicate determinations of the hydrogen-ion concentration.

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[1] The haemoglobin used in these experiments was prepared from the corpuscles of oxalated horse blood, obtained through the kindness of the Mulford Biological Laboratories.

A typical experiment was carried out as follows. Several collodion sacs were filled with a freshly prepared 1 to 2 % solution of crystalline oxyhaemoglobin and dialysed against distilled water previously boiled and saturated with oxygen. The osmometer flask was loosely stoppered, so that sufficient carbon dioxide entered to make the outer fluid somewhat more acid than p_H 7·0. Most of the osmometers were kept in the ice box at about 2°. The outer fluid was renewed several times daily until the reaction remained constant and the osmotic pressure of the haemoglobin solution did not change. The osmotic pressure of a 1 % solution was about 10 mm. Hg, which agrees quite well with the calculated pressure using 16,600, the commonly accepted molecular weight of haemoglobin, as determined by several methods [Gamgee, 1898; Hüfner and Gansser, 1907; Barcroft and Hill, 1910].

Replacing the distilled water with weak acetic acid of p_H 3·8 to 4·0 (about 0·001 *M*) resulted in a four- to six-fold increase in the osmotic pressure. The acetic acid was renewed once or twice a day until the reaction of the outer fluid (tested colorimetrically) remained constant (one to two days). The elimination of the inner core of liquid in the osmometer by means of a glass float permitted the system to come to equilibrium rapidly. To be certain that equilibrium was obtained, however, most of the osmometers were allowed to stand for 3 or 4 days. The reactions of the haemoglobin solution and the acetic acid outer fluid were then determined by means of the hydrogen electrode. As a check on the permeability of the sacs to acetic acid, a sac containing distilled water (p_H 6·0–7·0) was dialysed against acetic acid of p_H 4·0. The p_H of the fluid inside the sac became 4·0 in less than 24 hours.

At the end of an experiment the haemoglobin from one or two of the osmometers was filtered and the percentage haemoglobin determined by evaporating a sample to dryness. It was always found that during the experiment some of the haemoglobin had been precipitated out of the solution, the reduction in concentration being 15 % or less. With the weak solutions used, this decrease in concentration introduced only a slight error in the comparison of osmotic pressure readings at the beginning and end of an experiment.

No attempt was made to determine the amount of conversion of oxyhaemoglobin into reduced haemoglobin and methaemoglobin. In the previous paper several reasons were given for believing that, under the conditions of these experiments, splitting of the haemoglobin molecule did not occur.

Typical results of the acetic acid experiments in four osmometers (two different samples of oxyhaemoglobin being used) are as follows:

Concentration of Hb solution %	Osmotic pressure of Hb solution		p_H of acetic acid (outer fluid) at end of experiment	p_H of Hb (inner fluid)	
	Against water mm. Hg	Against acetic acid mm. Hg		Found at end of experiment	Calculated on basis of Donnan equation
1·7	16	70	3·8	5·2	5·1
1·7	15	71	3·9	5·2	5·4
1·2	10	50	4·0	5·5	5·3
1·2	8	50	4·0	5·6	5·5

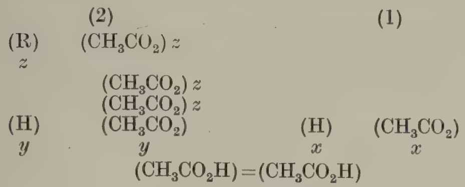
It will be seen from an examination of the fourth and fifth columns of the table that, at the end of such an experiment as has been described, the haemoglobin solution was considerably less acid than the acetic acid outer fluid, at a time when the osmotic pressure had increased four- to six-fold. This increased pressure might conceivably be due to one of a number of different causes, but, as was shown in the previous paper [Wilson, 1923], in every case, except that of the formation of haemoglobin acetate, the acetic acid would distribute itself so as to give the same hydrogen-ion concentration on each side of the membrane. In the case of the formation of haemoglobin acetate, however, the acetate ions therefrom would prevent an equal distribution of ions from the acetic acid, in accordance with Donnan's theory of membrane equilibrium. When the data are studied quantitatively, using the Donnan equation, it is found that the haemoglobin solution inside the osmometer should be less acid than the outer fluid, as was experimentally found to be the case. The increased osmotic pressure would be due to the haemoglobin ions and the acetate ions of the haemoglobin acetate (three to five acetate ions being necessary to account for the four- to six-fold increase in osmotic pressure). The counter osmotic pressure in these experiments is too slight to influence the calculations.

The calculations are necessarily rough, due to certain shortcomings in the data. The osmotic pressure readings are not quite as uniform as one would wish and the concentration of the haemoglobin undergoes a certain diminution during the course of the experiment. In postulating three to five acetate radicles for each haemoglobin radicle to account for the osmotic pressure change, it is assumed that there is no aggregation of the haemoglobin. If there were aggregation the ratio of acetate radicles to haemoglobin radicles would have to be larger.

Despite these sources of error, columns 5 and 6 in the table show that there is a relatively close agreement between the calculated and experimental values.

The calculations for the first of the experiments listed in the table above are given herewith.

Letting R be the cation of haemoglobin acetate, (1) outer fluid, (2) inner fluid, and x, y and z ion concentrations, we have the following scheme when equilibrium in the osmometer is attained.



$$\begin{array}{llllll}
 & (2) & & & (1) & \\
(R) & (CH_3CO_2)\,z & & & & \\
z & & & & & \\
 & (CH_3CO_2)\,z & & & & \\
 & (CH_3CO_2)\,z & & & & \\
(H) & (CH_3CO_2) & & (H) & (CH_3CO_2) & \\
y & y & & x & x & \\
 & \multicolumn{4}{c}{(CH_3CO_2H) = (CH_3CO_2H)} & \\
\end{array}$$

$$y\,(y+3z)=x^2.$$

When the p_H of the outer fluid is 3·8, $x = 0{\cdot}00016\ N$. A 1·7 % haemoglobin solution (molecular weight of haemoglobin 16,600) is 0·0010 M, hence

$$z = 0{\cdot}0010\ M.$$

Substituting in

$$y\,(y + 3z) = x^2,$$
$$y\,(y + 0{\cdot}003) = 0{\cdot}0000000256,$$
$$y^2 + 0{\cdot}003y = 0{\cdot}0000000256.$$

y^2, being infinitesimal as compared with the other values, may be disregarded. Hence

$$y = 0{\cdot}0000085.$$

The hydrogen-ion concentration (y) of the inner fluid which contains the haemoglobin acetate is, therefore, 0·0000085 N ($p_H = 5{\cdot}1$), while the hydrogen-ion concentration (x) of the outer fluid which contains only acetic acid is 0·00016 N ($p_H = 3{\cdot}8$). The experimental value, as seen in the table, is 5·2, instead of the calculated 5·1.

The results strongly indicate that the Donnan equilibrium was operating in these experiments and that haemoglobin acetate is formed when acetic acid is used as outer fluid in an osmometer with haemoglobin on the inside.

This conclusion can doubtless be extended to the formation of other acid salts of haemoglobin, under similar conditions, the hydrogen-ion concentration apparently being the determining factor. In the previous paper carbonic and lactic acids of the same p_H as the acetic acid used in these experiments gave identical osmotic pressure results.

SUMMARY.

When haemoglobin is dialysed in an osmometer against acetic acid, there results a four- to six-fold increase in osmotic pressure above the value against distilled water. When equilibrium is reached the hydrogen-ion concentration of the haemoglobin solution is found to be considerably less than that of the acetic acid outer fluid. Such an unequal distribution of hydrogen ions may be explained by Donnan's theory of membrane equilibrium. The existence of a Donnan equilibrium under these conditions proves that haemoglobin acetate has been formed. The formation of haemoglobin acetate adequately explains the increased osmotic pressure.

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XIII. THE TYROSINASE-TYROSINE REACTION. II.

THE THEORY OF DEAMINATION.

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The action of tyrosinase on tyrosine by which the latter is converted into pigmented substances has been studied by many, and various theories have been evolved to explain the mechanism of the oxidation processes. All these theories, however, are based on the assumption that deamination of the tyrosine takes place. Chodat and Schweizer [1913] investigated the action of tyrosinase on glycine and stated that among the end-products were formaldehyde, CO_2 and NH_3. Bach [1914] applied this idea of deamination of the amino-acid to the action of the enzyme or mixture of enzymes on tyrosine and suggested that the first action of tyrosinase on tyrosine could be represented as

$$\underset{\text{Tyrosine}}{OH\cdot C_6H_4\cdot CH_2\cdot CH\,(NH_2)\cdot COOH} + H_2O + \underbrace{A}_{\substack{\text{Hydrogen}\\ \text{acceptor}}} \rightarrow \underset{p\text{-Hydroxyphenylacetaldehyde}}{OH\cdot C_6H_4\cdot CH_2\cdot CHO} + NH_3 + CO_2 + AH_2$$

This reaction Bach attributed to an amino-acidase which splits up the tyrosine molecule into substances which are easily oxidised by the other enzyme component of tyrosinase, the phenolase (or peroxidase + H_2O_2). This action of the amino-acidase may be represented according to Bach as analogous to Strecker's reaction and results in deamination accompanied by or followed by decarboxylation.

$$\underset{\text{Tyrosine}}{OH\cdot C_6H_4\cdot CH_2\cdot CH\,(NH_2)\cdot COOH} + O\ (\text{active}) \rightarrow \underset{p\text{-Hydroxyphenylpyruvic acid}}{OH\cdot C_6H_4\cdot CH_2\cdot CO\cdot COOH} + NH_3\ \ldots(1)$$

$$OH\cdot C_6H_4\cdot CH_2 . CO\cdot COOH + (\text{Carboxylase}) \rightarrow \underset{p\text{-Hydroxyphenylacetaldehyde}}{OH\cdot C_6H_4\cdot CH_2\cdot CHO} + CO_2\ \ldots\ldots(2)$$

Similar views have been expressed by other workers. Folpmers [1916] carried out experiments which he interpreted as indicating that tyrosinase consists of two enzymes, a deaminising enzyme which converts tyrosine into *p*-hydroxyphenylacetaldehyde and a second enzyme which causes the further oxidation of the aldehyde (or its condensation product with ammonia) to form the black pigment melanin. Onslow [1923] accepts the view of Bach that the first step is the deamination of the tyrosine, followed by the further oxidation of these products and condensation with NH_3 to form melanin, and suggests that the reactions proceed as in equations (1) and (2). Haehn [1919] considers that tyrosinase consists of at least three different enzymes, one of which is a deaminase. Keeble, Armstrong and Jones [1913] have used the deamination theory in explaining pigment formation in plants. Abderhalden and Sickel

[1923] consider that the first stage in the tyrosinase-tyrosine reaction is a hydrolytic deamination accompanied by loss of CO_2. Thus all these theories involve the preliminary deamination of the tyrosine to form products which are subsequently oxidised (in some cases with condensation with NH_3) to form melanin. The experimental evidence supporting this view of deamination does not appear to be very weighty and the theory rests on a few isolated observations. Chodat and Schweizer [1913] identified H.CHO and NH_3 as products of the action of tyrosinase on glycine, and state that benzaldehyde is formed from phenylglycine, and acetaldehyde from alanine by the action of tyrosinase. These experiments have been repeated by Happold and Raper with negative results [1925]. Bach [1914] to support his theory of deamination attempted to show that *p*-hydroxyphenylacetaldehyde in the presence of the requisite amount of ammonia is oxidised by tyrosinase to the same end-products as those obtained in the tyrosinase-tyrosine reaction. In view of the difficulty in obtaining this aldehyde, Bach substituted the next lower member of this series, *p*-hydroxybenzaldehyde and claimed that by the action of peroxidase and H_2O_2 on this aldehyde in the presence of ammonia, colour changes similar to those of the tyrosinase-tyrosine reaction were obtained. These results, the rapid reddening of the solution followed by darkening to a violet-black colour, were not obtained when a phenolase was substituted for the peroxidase and H_2O_2 system, and this is attributed to the greater inhibitory action of NH_3 on phenolase. The only other evidence in support of the deamination theory appears to be that offered by Folpmers [1916], who isolated the *p*-nitrophenylhydrazone of benzaldehyde from a reacting mixture containing phenylglycine and plant juices, and an analogous hydrazone in extremely small quantities (too small for identification) when tyrosine was used.

The tyrosinase-tyrosine reaction may be represented as taking place in the following manner [Raper and Wormall, 1923]:

Tyrosine + (Tyrosinase) + O → Red pigment ... ... (3)
Red pigment → Colourless substance (probably an intramolecular change—spontaneous but greatly accelerated by warming) ... ... (4)
Colourless substance + O → melanin (accelerated by the phenolase of tyrosinase) (5)

If the reaction is carried out in slightly acid solution (p_H 6·0), the red substance is the main pigmented product formed during the first 6 or 8 hours, during which time from 50 % to 75 % of the tyrosine may be oxidised. This red substance is the first visible sign of oxidation although it may not be the first intermediate product in the oxidation process. On the assumption that deamination takes place, according to equation (1), the oxidation of tyrosine by tyrosinase will result in the liberation of an equivalent amount of NH_3 which should pass into solution. If no increase in the ammonia content of the reacting mixture takes place as the reaction proceeds, such a result would admit of two possibilities: (*a*) that no deamination of tyrosine occurs or (*b*) that deamination takes place but is immediately followed by the recombination of the NH_3 with the products of deamination (and decarboxylation) to form a substance which in this case must be the red pigment. The second conjecture appears improbable and would involve the assumption that the deaminised products in the presence of NH_3 are rapidly converted by tyrosinase into the red pigment. That this assumption is incorrect will be shown later and thus the non-liberation of NH_3 as the reaction proceeds is not in favour of the theory of deamination.

In the present work, tyrosine solutions buffered at p_H 6·0 were acted upon by tyrosinase from potatoes and the ammonia content of the reacting mixture determined at intervals during the oxidation of at least two-thirds of the tyrosine present. No increase in the ammonia content of these solutions took place at any time, although the amount of NH_3 which should have been liberated according to equation (1) is quite appreciable. Additions to special samples of the solution of one-quarter of the amount of NH_3 which should theoretically be produced, calculated from the amount of tyrosine oxidised, resulted in these added quantities being estimated almost quantitatively, and thus satisfactory conditions appear to have been obtained. These ammonia estimations were carried out by the permutite filtration method [Whitehorn, 1923] for this method is of special application to slightly acid solutions. The ordinary methods for estimating NH_3 involve adding an excess of alkali and driving off the NH_3 into an acid solution and could not be applied in this instance, as the addition of the alkali would result in the rapid blackening of the solution. Thus the objection could then have been made that the free NH_3 in the solution had condensed after the addition of the alkali, with the products of tyrosine deamination, to form melanin. This inability to obtain any increase in the ammonia content of the reacting tyrosinase-tyrosine solution is strong evidence against the theory of deamination.

The property of permutite in removing all ammonia from neutral or slightly acid solutions may be utilised in obtaining qualitative evidence against the theory of deamination. The view that the final product, melanin, contains nitrogen is generally accepted and has been confirmed in the present work, but according to Bach [1914] this product is formed as the result of oxidation of the deaminised tyrosine accompanied by condensation with NH_3. If this is true, the removal of NH_3 from the reacting mixture should prevent the darkening of the solution, *i.e.* formation of melanin. It has been found however that no diminution in the production of melanin can be effected by shaking up the following solutions with permutite or filtration of these solutions through a layer of permutite: (1) the tyrosinase-tyrosine reaction mixture, (2) the clear red solution free from enzyme and (3) the colourless solution free from enzyme. Thus the removal of all the ammonia from these solutions does not inhibit the production of the final product melanin. It thus appears unlikely that ammonia is split off at any stage in the reaction.

From equation (1) it will be seen that *p*-hydroxyphenylpyruvic acid will be the first product or first non-nitrogenous product of the deamination of tyrosine and for this theory to hold good it is necessary that tyrosinase should act on p-hydroxyphenylpyruvic acid in the presence of the requisite amount of ammonia to form the same end-products as the tyrosinase-tyrosine reaction. This acid has been prepared and it has been found that tyrosinase has no apparent action on it alone or with the addition of the calculated amount of NH_3. In addition, no increase in the amount of red pigment, colourless substance or melanin could be produced by adding this acid, with or without the addition of the calculated quantity of NH_3, to a reacting tyrosinase-tyrosine solution. This inability of tyrosinase to oxidise one of the intermediate products of the reaction according to the deamination theory, appears to be conclusive evidence against this theory.

The final product of the tyrosinase-tyrosine reaction, melanin, can be obtained in a comparatively pure state by the method described below, and prepared in this way, it is found to be a nitrogenous substance with a somewhat higher percentage of nitrogen than tyrosine. From the facts mentioned above, (*a*) the non-liberation of ammonia during the reaction, (*b*) the non-effect of removing any free ammonia from the reacting mixture on the production of red substance, colourless substance or melanin, and (*c*) the inability of tyrosinase to oxidise p-hydroxyphenylpyruvic acid in the presence of NH_3, the conclusion must be drawn that no deamination takes place in the tyrosinase-tyrosine reaction. Further, since the final product, melanin, contains approximately the same percentage of nitrogen as tyrosine and as the intermediate products have certain properties which lead to the supposition that they are nitrogenous, it is suggested that all the intermediate products and final product contain nitrogen and that at no stage of the reaction is nitrogen split off in the form of ammonia.

EXPERIMENTAL.

In all the experiments described below except where the contrary is stated, dialysed potato juice prepared in the manner previously described [Raper and Wormall, 1923] was used as the source of tyrosinase. All the reactions were carried out in phosphate buffered solutions since the p_H of the solution has a very marked influence on the nature and quantity of the products obtained.

Ammonia content of the reacting tyrosine-tyrosinase mixture.

For this determination, the tyrosine solution was buffered at p_H 6·0, this reaction being considered most suitable for the purpose. In alkaline solutions and to a less extent at p_H 7·0 the red substance is rapidly converted into melanin, and according to the theory of Bach this melanin formation is due to the recombination of the deaminised fraction with ammonia and to condensation processes. Thus the best conditions for the detection and estimation of the liberated ammonia will be those in which the amount of melanin formed is at a minimum, while the amount of tyrosine oxidised is at or near the maximum. These conditions are best satisfied by allowing the enzyme action to proceed in slightly acid solutions, and at p_H 6·0 the velocity of the oxidation is not greatly inferior to that at p_H 7·0 or 8·0 [Raper and Wormall, 1923].

Dialysed potato juice was added to the tyrosine solution, toluene added and a constant current of air drawn through this and a control solution containing no tyrosine, as previously described for the determination of the velocity coefficients. Four samples of 60 cc. each were withdrawn from each of the two solutions and half of these samples were heated to convert the red pigment where present into the colourless substance. The ammonia content of all these samples was then determined by the method of Whitehorn [1923], which involves the filtration of the solution through a layer of permutite, the liberation of the NH_3 by the addition of alkali to the permutite and nesslerisation. No increase in the ammonia present in the reacting tyrosine-tyrosinase solution could be observed at any time and since about two-thirds of the tyrosine present was oxidised during the time of observation, the amount of NH_3 liberated according to the deamination theory would have been appreciable.

Method of ammonia estimation.

Each sample (60 cc.) was filtered slowly through about 5 cc. of finely granular permutite contained in a $CaCl_2$ tube, the permutite having previously been washed with 2 % acetic acid to neutralise any alkali present and several times with distilled water. When the solution whose ammonia content was to be determined had filtered through, the permutite was washed five times with 10–20 cc. of distilled water and finally rinsed with the minimum quantity of water into a 100 cc. graduated flask. 5 cc. of 10 % NaOH were added to each suspension of permutite containing ammonia, the solution shaken for a minute and 15 cc. of modified Nessler's solution (Bock and Benedict formula) added. The solutions were allowed to stand for 15 minutes, centrifuged when necessary and estimated colorimetrically against a standard prepared as follows: 2 cc. of a standard NH_4Cl solution (5 cc. contain 1 mg. NH_3) + 20 cc. dist. H_2O + 5 cc. 10 % NaOH + 15 cc. mod. Nessler's solution. The solution was shaken, allowed to stand for 15 minutes and then made up to 100 cc.

Duplicate estimations were carried out after each interval of time and "test estimations" were also made by adding to special samples of the reaction mixture 2 cc. of the standard NH_4Cl solution (2 cc. contain 0·40 mg. NH_3). The procedure for these test estimations was the same as that for the ordinary samples, and these added amounts of NH_3 were estimated almost quantitatively.

Tyrosine estimations.

These were carried out on 20 cc. samples withdrawn from the solutions at the commencement of the reaction and after $7\frac{1}{2}$ hours and the method used was the bromination method previously described. The results show that approximately two-thirds of the tyrosine was oxidised during the experiment.

Solution A. 1000 cc. phosphate buffered 0·05 % tyrosine solution (p_H 6·0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution B. 1000 cc. phosphate buffered water (p_H 6·0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution A (red) refers to the samples of the reacting mixture which were used unchanged for the estimations.

Solution A (colourless) refers to samples which had been boiled to convert the red pigment into the colourless substance. This conversion was brought about by adding to the sample 1 cc. $N/10$ HCl, boiling, filtering and finally adding 1 cc. $N/10$ NaOH.

Results. Ammonia content of the samples in mg. per 60 cc.

Solution A (red)	0·16	0·17	0·17	0·16
Solution A (colourless) ...	0·17	0·18	0·20	0·18
Solution B (unboiled) ...	0·15	0·17	0·16	0·17
Solution B (boiled) ...	0·15	0·18	0·16	0·16
Tyrosine in Solution A (%)	0·045	—	—	0·013
Time (hrs)	0	3	5	7½

The amount of NH_3 which would have been liberated by the deamination of the tyrosine which was oxidised in 7½ hours is 1·80 mg. per 60 cc., and as less than a quarter of this amount, if added to a sample, was estimated in full, it appears inconceivable that deamination or the liberation of ammonia takes place during the action of tyrosinase on tyrosine.

The action of tyrosinase on p-*hydroxyphenylpyruvic acid.*

This acid has been prepared by the method used by Neubauer [1909], but the filtrate from the benzoic acid was immediately extracted with successive quantities of ether until almost all of the keto acid had been extracted, as shown by the inability of the neutralised aqueous solution to give more than a faint green colour with dil. ferric chloride solution. The ethereal solution containing the *p*-hydroxyphenylpyruvic acid was distilled under reduced pressure to remove the solvent, the residue dissolved in the minimum quantity of hot alcohol and poured into cold water. A white crystalline ppt. of the keto acid separated, was filtered off, washed with a little benzene and dried *in vacuo*. In some cases the acid was practically pure but in other cases had to be recrystallised from hot water (M.P. 220°). A further quantity of the acid can be recovered from the mother liquor. Solutions containing 0·05 % of this keto acid were then prepared and buffered at p_H 6·0, 7·0 and 8·0. These solutions were then acted upon by potato juice both with and without the amount of NH_3 (as NH_4Cl) theoretically required.

Controls were also set up at each p_H, using only the phosphate buffers and potato juice. The whole of the experiment was then repeated using dialysed potato juice as the source of tyrosinase and the results were exactly the same as those quoted below.

Experiments were then carried out to see whether the addition of *p*-hydroxyphenylpyruvic acid together with the theoretical amount of NH_3 to reacting tyrosine-tyrosinase solutions would increase the yield of red pigment, colourless substance or melanin. No increase in the amount of either of the intermediate compounds or of the final product took place.

Observations.

Solutions used: I. 0·05 % phosphate buffered tyrosine solution (p_H 6·0, 7·0 and 8·0).
II. 0·05 % phosphate buffered *p*-hydroxyphenylpyruvic acid solutions (p_H 6·0, 7·0 and 8·
1·5 % NH_4Cl solution. Fresh and dialysed potato juice.

			1 hour	4½ hours	36 ho
1.	100 cc. Solution I (p_H 6·0)	+10 cc. potato juice	Red	Red	Black
2.	100 ,, ,, II (p_H 6·0)	+10 ,, ,, ,,	No change	No change	No cha
3.	100 ,, ,, II (p_H 6·0)	+10 ,, ,, ,, +1 cc. 1·5 % NH_4Cl	No change	No change	No cha
4.	100 ,, ,, I (p_H 7·0)	+10 cc. potato juice	Red	Black-red	Black
5.	100 ,, ,, II (p_H 7·0)	+10 ,, ,, ,,	No change	No change	No cha
6.	100 ,, ,, II (p_H 7·0)	+10 ,, ,, ,, +1 cc. 1·5 % NH_4Cl	No change	No change	No cha
7.	100 ,, ,, I (p_H 8·0)	+10 cc. potato juice	Red-black	Black	Black
8.	100 ,, ,, II (p_H 8·0)	+10 ,, ,, ,,	No change	No change	No cha
9.	100 ,, ,, II (p_H 8·0)	+10 ,, ,, ,, +1 cc. 1·5 % NH_4Cl	No change	No change	No cha

These results lead to the conclusion that *p*-hydroxyphenylpyruvic acid is not an intermediate product in the oxidation of tyrosine by tyrosinase and thus the view that deamination occurs according to equations (1) and (2) appears to be untenable.

The preparation and nitrogen content of the melanin.

2 g. of tyrosine were dissolved in 4 litres of water, 400 cc. of fresh potato juice added and the reaction of the solution adjusted to p_H 6·0. The solution was then warmed to 37° and stirred vigorously in a wide vessel. When the intensity of the red colour reached a maximum and the solution was beginning to darken slightly, usually after 5–6 hours, the stirring was stopped. The solution was transferred to a 5-litre flask, 40 cc. of 10 % acetic acid added and the liquid boiled as quickly as possible. After being thoroughly cooled, the solution containing the coagulated proteins was filtered, and the filtrate allowed to stand overnight after being made definitely alkaline with 100 cc. 10 % Na_2CO_3. On the addition of the alkali the colourless solution rapidly darkened and the formation of the melanin was accelerated by shaking with air. The melanin obtained in this way could not be filtered off, but was obtained in the precipitated condition by adding 200 cc. 10 % acetic acid and boiling the solution. The melanin was filtered off, leaving a clear solution and was purified as far as possible by boiling with dil. HCl, then with successive quantities of water, and washing with hot water. It was dried on porous plates *in vacuo* over H_2SO_4. Examined under the microscope it appeared to be amorphous and was hard enough to be powdered finely. Nitrogen was present in considerable quantity according to the qualitative test (fusion with Na, etc.), but no sulphur. The absence of sulphur is evidence in favour of the view that the melanin is free from nitrogenous substances derived from the

potato juice, for the proteins of this juice have been found to contain sulphur. Four different samples of melanin were prepared by the above method, the yield being 0·5 g. to 0·8 g. melanin from 2 g. tyrosine, and the nitrogen content determined by the Kjeldahl method. The results showed that 8·43 %, 8·30 % and 8·47 % of nitrogen were present in three of the preparations by the Kjeldahl method and 8·65 % by the Dumas method. This is rather higher than the percentage of N in tyrosine (7·73 %), and is probably due to the fact that although oxygen has been absorbed by the tyrosine, molecules of water have been removed in the condensation processes, with resulting slight increase in the percentage of nitrogen present.

SUMMARY AND CONCLUSIONS.

1. Evidence against Bach's theory of deamination of the tyrosine has been obtained in the following manner and appears to be a conclusive refutation of this view.

(*a*) A reacting tyrosinase-tyrosine solution shows no increase in ammonia content during the oxidation of at least two-thirds of the tyrosine present.

(*b*) Removal of all the ammonia initially present in the solutions or formed by any part of the oxidation process does not affect in any way the formation of the intermediate products, *i.e.* the red and colourless substances, or the final product melanin.

(*c*) Tyrosinase has no action on solutions of *p*-hydroxyphenylpyruvic acid either with or without the necessary ammonia for recondensation.

2. The final product of the tyrosinase-tyrosine reaction, melanin, is a nitrogenous compound with a slightly higher percentage of nitrogen than tyrosine (8·40 % against 7·73 % N in tyrosine).

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XIV. THE TYROSINASE-TYROSINE REACTION. III.

THE SUPPOSED DEAMINISING ACTION OF TYROSINASE ON AMINO ACIDS.

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For some years it has been generally accepted that the enzyme tyrosinase will bring about the oxidation of amino acids with the production of ammonia. This view was first suggested by Chodat and Schweizer [1913] to explain the production of formaldehyde and ammonia from glycine when it was submitted to the action of tyrosinase from potatoes. The process of deamination has been made the basis of a theory as to the components of the enzyme tyrosinase by Bach [1914], who accepts the view that tyrosinase has a deaminising action on tyrosine. Folpmers [1916] isolated a small amount of benzaldehyde as the *p*-nitrophenylhydrazone from the products of the action of tyrosinase on phenylaminoacetic acid, thus supporting the deamination hypothesis.

A study of the experimental evidence on which the deamination theory was based led us to the opinion, that certain of the experiments should be repeated and extended, and the results of this work are included in the present paper.

The tyrosinase used throughout this investigation was obtained by the dialysis of potato juice through moderately permeable collodion sacs. An attempt was made in the first instance to obtain from alanine, by the action of tyrosinase, the aldehyde corresponding to it, namely, acetaldehyde. This proved abortive although the enzyme preparations used were very active as shown by their action on tyrosine. It was then found that under the conditions of the experiments, in which free access of oxygen is a necessity, small traces of added acetaldehyde disappeared, probably by oxidation. Attempts to detect formaldehyde and benzaldehyde as products of the action of tyrosinase on glycine and phenylaminoacetic acid respectively were also negative. The search for aldehydes as products of the tyrosinase reaction was therefore discontinued and instead experiments were carried out in which the ammonia, if any, produced by the action of tyrosinase on amino acids could be estimated. The acids chosen were glycine, alanine, phenylaminoacetic, phenylalanine and tyrosine. The conditions were such that had any ammonia been produced it would have been estimated easily. These experiments also provided no evidence of deamination and in this respect agreed with the experiments of Raper and Wormall on tyrosine [1925] carried out by other methods.

Bach [1914] has suggested that after deamination the ammonia recombines with some other product of the enzyme reaction—possibly the aldehyde—and as this might conceivably lead to the non-detection of ammonia as a product of the reaction, a series of experiments was carried out to determine whether any change in the amino N content of the system took place when tyrosinase acted on glycine, alanine or phenylaminoacetic acid. The results were completely negative. In addition, measurements have been made of the oxygen taken up when tyrosinase acts on tyrosine, glycine and alanine under identical conditions. A considerable oxygen absorption was observed with tyrosine but none with the other two amino acids. We have thus come to the conclusion that potato tyrosinase, under the conditions in which the enzyme is very active, has no deaminising action on amino acids.

These results necessitated a re-examination of the experiments of Chodat and Schweizer in which they claimed to have obtained evidence of deamination of glycine by potato tyrosinase, by the detection of formaldehyde and ammonia as products of the reaction. On reading over the records of Chodat and Schweizer's experiments, it was noticeable that success was only achieved when *p*-cresol was added to the reacting system. This addition was made, according to Chodat and Schweizer, in order to prevent interaction of the ammonia and formaldehyde produced by the deamination process, since the uncertainty in detecting them when *p*-cresol was not present was attributed to their further reaction with each other.

On repetition of these experiments we have been able to confirm that when *p*-cresol is present along with glycine and tyrosinase, ammonia is liberated and there is a diminution in the amino-nitrogen content of the system.

The evidence in favour of formaldehyde production adduced by Chodat and Schweizer we have not been able to confirm. *p*-Cresol itself reduces ammoniacal silver nitrate solution so that in its presence a reduction of this reagent cannot be accepted as evidence of the presence of formaldehyde, a point seemingly overlooked by Chodat and Schweizer.

Schryver's reaction for formaldehyde—the second reaction which they used—has failed in our hands to show the presence of formaldehyde when tyrosinase acts on glycine. This reaction will detect with ease 1 part in 10,000 of formaldehyde so that if it is produced even in traces it should have been detected. We are led therefore to the conclusion that the deamination found by Chodat and Schweizer was due to the presence of *p*-cresol and tyrosinase and not to tyrosinase alone, and as Chodat has shown that the latter attacks the *p*-cresol, it is probable that the deamination is produced by some oxidation product of *p*-cresol acting on the amino acid.

It was of interest to compare the action of other phenolic and related substances with that of *p*-cresol, and this has been done with phenol, resorcinol, quinol, catechol, *p*-benzoquinone and *o*-benzoquinone.

Only those phenols which are likely to give an *ortho*-dihydric-phenol or *ortho*-quinone derivative on oxidation gave positive results in the deamination experiments. Under the conditions in which the experiments were carried out, even in the absence of tyrosinase, *o*-quinone produced deamination. This suggests that the phenols which were found to be active display this activity because they yield *o*-quinone or a derivative of it (*e.g.* 3·4 toluquinone from *p*-cresol), on oxidation by tyrosinase and that this then reacts with the amino acid.

It should be noted that M. W. Onslow [1923] has suggested that tyrosinase is a mixture of enzymes; a water splitting enzyme (reductase or deaminase), a carboxylase and an oxydase (consisting of an oxygenase, a dihydroxy substance and a peroxydase), and that she suggests that *p*-cresol is oxidised by traces of active oxygen to 3·4-dihydroxytoluene, but there is no assumption of the formation of an *o*-quinone which reacts with the amino acid and causes deamination.

The observations recorded in this paper indicate that tyrosine is acted on by tyrosinase, not because it is an amino acid but because it possesses a phenolic hydroxyl group. Phenylalanine, which only differs from tyrosine in this particular, is not oxidised by tyrosinase. It seems likely therefore that a preliminary stage in the oxidation of tyrosine by tyrosinase may be the formation of an *o*-quinone derivative which then undergoes an intramolecular reaction with the side chain containing the amino group, or reacts with another molecule of tyrosine. The amino group is, however, not split off as ammonia, as it is when *o*-quinone attacks other amino acids.

EXPERIMENTAL.

In the experiments with tyrosinase described in this section, a phosphate buffer mixture adjusted to p_H 7 was used and toluene was added as an antiseptic.

I. *The Alleged Aldehyde Formation due to the Action of Tyrosinase on Amino Acids.*

(*a*) *From alanine.* For the detection of acetaldehyde, *p*-nitrophenylhydrazine was used in acetic acid solution. Two large flasks *A* and *B* were taken and into each was placed a mixture of 100 cc. dialysed potato juice, 100 cc. of the buffer solution and 10 cc. of toluene, in addition 1 g. of alanine was placed in *A*. The two flasks were left at room temperature for 24 hours, after which the contents were made just acid to Congo red by the addition of phosphoric acid and distilled.

The distillate was first caused to pass through a percolator packed with broken ice, after which it was collected in two separate portions, 25 cc. in volume, in a receiver, surrounded by melting ice, which contained the maximum theoretical amount of a *p*-nitrophenylhydrazine solution necessary for the conversion of any aldehyde formed into the *p*-nitrophenylhydrazone. A very faint turbidity was produced by the distillate obtained from *A*, while that from *B* produced no change.

This failure to detect acetaldehyde in the products of the reaction led to the consideration of the possibility under the conditions of the experiment of

(1) condensation of the aldehyde with itself,
(2) loss of aldehyde by oxidation and
(3) a loss of aldehyde by volatilisation.

To test these possibilities the following experiments were undertaken:

(1) The above experiment was repeated with the phosphate buffer adjusted to p_H 6·5 so as to prevent the aldol condensation of acetaldehyde. Again no acetaldehyde was detected as a reaction product, thus ruling out autocondensation as an explanation of its possible disappearance from the system.

(2) Two 2-litre flasks were taken and into each was placed a mixture containing 50 cc. dialysed potato juice, 50 cc. buffer solution and 0·05 cc. acetaldehyde; the one was stoppered and the other unstoppered. Two similar flasks were set up at the same time as controls, water displacing the enzyme preparations previously used. After standing for 24 hours each flask was tested for acetaldehyde by the *p*-nitrophenylhydrazine reaction as previously described. There was a greater amount of hydrazone formation in the experiments in which water had taken the place of the enzyme and also in the case of the stoppered flasks as compared with the unstoppered; the greatest difference was, however, *small* and it was concluded from the results that while the loss of acetaldehyde due to possible oxidation was by no means negligible, that due to evaporation could be ignored.

(*b*) *From glycine.* With a view to testing whether formaldehyde was produced from glycine by the action of tyrosinase, 0·25 g. of glycine dissolved in 50 cc. of buffer solution was mixed with 50 cc. of dialysed potato juice and 10 cc. of toluene and exposed to the air in a 2-litre flask for 24 hours side by side with a control which contained no glycine. At the end of the period portions tested with Schryver's reagent gave no formaldehyde reaction and no formaldehyde could be detected in the distillate collected over ice in the manner previously described for acetaldehyde.

(*c*) *From phenylaminoacetic acid.* Folpmers' experiment on the formation of benzaldehyde has also been carefully repeated with negative results. 0·2 g. of phenylaminoacetic acid was placed in a mixture of 25 cc. dialysed potato juice, 25 cc. buffer solution and 10 cc. toluene; a similar control with water replacing the enzyme was set up. After 24 hours' exposure to the air no benzaldehyde formation could be demonstrated in either case. The phenylaminoacetic acid used in this experiment was prepared in the usual way by the hydrolysis of its nitrile hydrochloride and was carefully purified by recrystallisation from dilute acetic acid. It seemed possible that a contamination of the phenylaminoacetic acid with its nitrile as a result of incomplete hydrolysis might have accounted for Folpmers' results, so to test this 1 g. of the nitrile hydrochloride was subjected to the action of tyrosinase for 24 hours in the presence of air and the solution subsequently acidified and distilled.

From the distillate 0·42 g. of the *p*-nitrophenylhydrazone of benzaldehyde was obtained which was practically pure after one crystallisation. It appears therefore that the apparent deamination of phenylaminoacetic acid demonstrated by Folpmers may possibly have been due to the contamination of the phenylaminoacetic acid with its nitrile. In his paper, however, Folpmers does not state the source of the phenylaminoacetic acid which he used.

II. *The Alleged Ammonia Formation.*

The method of detecting ammonia formation was based on Folin's method of estimating ammonia in urine. The apparatus, which was in duplicate, consisted of three bottles, *A*, *B* and *C*, which were connected in series and air was aspirated through both sets by the same pump; *A* contained 20 % H_2SO_4 so that any air drawn through it would be ammonia free, *B* was the reaction bottle in which the enzyme-buffer mixture was placed and *C* the absorption bottle, contained 100 cc. of $N/50$ H_2SO_4.

The whole having been connected up, 10 cc. of toluene, 25 cc. of dialysed potato juice and 25 cc. of the buffer solution containing a definite amount of the amino acid were added to the reaction bottle *B*; a control mixture without the amino acid being placed in the reaction bottle of the duplicate series.

By means of a water pump the whole was aerated gently for 24 hours. At the end of this time about 4 g. of Na_2CO_3 and a few drops of capryl alcohol were added to each reaction bottle, and any free ammonia in the reaction bottle was drawn over into the respective absorption bottles.

The figures represent the cc. of $N/10$ NaOH used in the titration of the acid in the absorption bottles. Any formation of ammonia in the direct experiment would cause this figure to be lower than the figure from the control bottle. In no case was deamination observed.

	Experimental		Control	
	Readings	Average	Readings	Average
Alanine, 0·25 g.	19·90 19·94 19·95 19·95	19·93	20·01 19·99 19·95 19·99	19·98
Glycine. 0·5 g.	19·96 19·89	19·93	20·00 20·01	20·01
Phenylalanine, 0·25 g.	19·98 19·98	19·98	19·90 20·04	19·97
Tyrosine, sat. sol.	19·95 19·97	19·96	20·02 20·04	20·03
Phenylaminoacetic acid, sat. sol.	19·85 19·95	19·90	19·85 19·95	19·90

The small differences observed are within the experimental error.

Study of the Amino Nitrogen Content of the System during the Period of Aeration.

The reaction and control mixtures were aerated in the same apparatus as that used in the ammonia determination, samples were withdrawn at intervals from both mixtures and the amino nitrogen content of these estimated by means of the micro van Slyke method.

The amino acids studied were alanine, glycine and phenylaminoacetic acid; in the case of the first two, 0·5 cc. of the enzyme-buffer mixture was used for each determination, in the case of the last 3·5 cc. were taken. The figures given are in cc. of nitrogen at N.T.P. The amino acid was dissolved in a mixture of equal parts, enzyme and buffer solutions.

Amino acid	Time hours	Experiment cc.	Control cc.	Difference cc.
Alanine: 0·4645 g. in 50 cc. enzyme-buffer solution	0	1·33	0·26	1·07
	24	1·39	0·26	1·13
	30	1·41	0·29	1·12
Glycine: 0·45 g. in 60 cc. enzyme-buffer solution	0	1·50	0·36	1·14
	2	1·51	0·37	1·14
	26	1·55	0·41	1·14
Phenylaminoacetic: 0·0349 g. in 60 cc. enzyme-buffer solution	0	0·53	0·26	0·27
	24	0·62	0·35	0·27
	26	0·62	0·35	0·27

These experiments show that the amount of amino nitrogen in the system in each case was unchanged after 24 hours or more.

As a useful comparison, figures were obtained showing the amount of tyrosine oxidised under the above conditions. The method adopted for the estimation was the modification of Miller's method devised by Raper and Wormall [1923]. A mixture of 60 cc. of the buffer solution containing 0·0264 g. of tyrosine and 60 cc. of dialysed potato juice having been divided into two parts 10 cc. of toluene were added to each, both portions being then aerated as in previous experiments. After 24 hours 0·0146 g. tyrosine was left, so that 0·0118 g. of tyrosine out of a possible 0·0264 g., or 44·7 %, had been oxidised.

Oxygen Absorption in Tyrosinase-Amino-Acid Mixtures.

If the alleged deamination of amino acids by tyrosinase is carried out by the same chemical processes which are involved when this enzyme acts on tyrosine then oxygen should be absorbed as it certainly is when tyrosine is the substrate. To investigate this experiments have been carried out in a Barcroft apparatus. In one bottle a buffered solution of the amino acid was used and in the other the buffer solution only. Toluene was present in each bottle and 0·2 cc. of enzyme was placed in the cup of each stopper. When equilibrium had been obtained with the bottles open to the air, they were connected to the manometer and the enzyme solution spilt into the bottles. Experiments were carried out with tyrosine (saturated solution in the buffer mixture), glycine and alanine, the amino acids all being used in approximately equimolecular quantities. No oxygen absorption was observed in 6 hours with glycine or alanine. There was, in fact, a slight increase in the case of alanine (12 mm.^{3}). With tyrosine a considerable oxygen absorption occurred, which at the end of 18 hours measured 0·1053 cc. These experiments indicate therefore that no oxidation of glycine or alanine takes place under conditions in which a considerable oxidation of tyrosine can be demonstrated. They do not support the view that tyrosinase exerts an oxidative deamination on amino acids in general.

III. *The Effect of the Addition of Phenols and Related Substances to the System Tyrosinase-Amino-Acid.*

Preliminary experiments showed that deamination and a diminution in the amino N content of the system took place when tyrosinase acted upon glycine in the presence of *p*-cresol. A systematic study was therefore made of the influence of *p*-cresol on other amino acids in the presence of tyrosinase and the investigation was extended to include other phenols and allied substances. The phenols, etc., used were phenol, catechol, resorcinol, quinol and *p*-benzoquinone. Of these only *p*-cresol, phenol and catechol produced deamination. The results are tabulated below. The method of experiment was as follows. The amino acid was dissolved in 50 cc. of the phosphate buffer solution, 50 cc. of dialysed potato juice were added and the solution divided into two equal portions. To one 10 cc. of a solution of the phenol or related substance were added and to the other, which served as a control, 10 cc. of water. Toluene was added as an antiseptic. The amino N was determined in a small sample from each at the beginning of the experiment and usually after 24 and 48 hours' gentle aeration at room temperature. Finally, any ammonia produced was liberated by adding solid sodium carbonate and blowing it over into *N*/50 sulphuric acid. The titration figures given in the last column but one give the amount of *N*/10 NaOH used in the titration of the excess of acid not neutralised by ammonia.

Phenol etc. used	Amino acid used		Amino N in cc.			cc. *N*/10 NaOH	NH_3 liberated mg.	Deamination
			0 hr.	24 hr.	48 hr.			
p-Cresol, 0·4 %	Alanine, 0·5 g.	Exp.	2·36	2·08	1·72	18·10	4·5	Positive
		Cont.	2·34	—	2·33	20·75		
	Phenylalanine, 0·3 g.	Exp.	0·93	0·83	0·73	18·50	3·8	Positive
		Cont.	0·94	—	0·86	20·75		
	Leucine, 0·5 g.	Exp.	1·63	—	0·91	17·67	5·0	Positive
		Cont.	1·59	—	1·60	20·65		
Phenol, 1·0 %	Glycine, 0·4 g.	Exp.	2·27	1·81	1·33	15·28	8·8	Positive
		Cont.	2·22	—	2·21	20·48		
	Alanine, 0·5 g.	Exp.	2·26	1·85	—	18·65	3·0	Positive
		Cont.	2·29	2·29	—	20·42		
Resorcinol, 0·5 %	Glycine, 0·3 g.	Exp.	1·90	1·95	—	19·81	0	Negative
		Cont.	1·78	1·76	—	19·76		
	Alanine, 0·5 g.	Exp.	2·51	2·46	2·43	19·71	0	Negative
		Cont.	2·52	2·49	2·52	19·71		
Quinol, 1·0 %	Glycine, 0·45 g.	Exp.	2·46	2·49	—	19·86	0	Negative
		Cont.	2·45	2·41	—	19·71		
	Alanine, 0·55 g.	Exp.	2·82	—	2·82	19·61	0	Negative
		Cont.	2·85	2·83	2·80	19·61		
	Phenylalanine, 0·3 g.	Exp.	1·11	1·16	—	19·99	0·17*	Negative
		Cont.	1·04	1·10	—	20·09		
p-Benzoquinone, 1·0 %	Glycine, 0·25 g.	Exp.	1·62	1·59	—	19·48	0·1*	Negative
		Cont.	1·61	1·60	—	19·54		
	Alanine, 0·5 g.	Exp.	2·64	2·66	—	19·95	0·17*	Negative
		Cont.	2·64	2·67	—	20·05		
	Phenylalanine, 0·25 g.	Exp.	0·99	0·98	—	19·49	0	Negative
		Cont.	0·98	1·00	—	19·50		
Catechol, 0·5 %	Glycine, 0·25 g.	Exp.	1·84	1·73	—	18·36	2·4	Positive
		Cont.	1·85	1·87	—	19·78		
	Alanine, 0·55 g.	Exp.	2·84	2·37	—	18·28	2·3	Positive
		Cont.	2·84	2·83	—	19·62		

* These small amounts are within the experimental error,

Since only those phenols which were likely to give an *ortho*-quinone on oxidation caused deamination to take place, experiments were carried out with *o*-quinone itself and it was found that this substance attacked amino acids, even in the absence of tyrosinase, causing a liberation of ammonia and diminution in the amino N content of the reacting system. The *o*-quinone was prepared by Willstätter and Müller's method [1908]. The quinone is very unstable and two methods were used to demonstrate its action. In the first the quinone obtained from 0·25 g. catechol was thrown down from its solution in ether by the addition of light petroleum, washed with the latter solvent and then redissolved in ether. This solution was added to the solution of the amino acid (0·175 g. glycine in 60 cc. of the buffer mixture) and a similar solution to which no quinone was added served as a control. Aeration was carried out for 24 hours. The initial amino N figures were 2·01 and 1·99 cc. for the experiment and 2·02 and 1·99 for the control; the final figures were 1·91 and 1·90 cc. for the experiment and 2·00 and 1·99 cc. for the control. The $N/10$ NaOH used for the acid in the ammonia absorption bottle was 18·88 cc. for the experiment and 19·58 cc. for the control. Both these results therefore provided satisfactory evidence of deamination. In the second experiment, the quinone from 0·5 g. catechol was precipitated directly from the ether in the reaction bottle and was not further purified, hence it would contain a small amount of catechol. The amino acid solution was added directly to this and 0·25 g. catechol was added to the control solution in order to allow for any possible deamination due to catechol alone. An experiment with 0·2 g. of glycine gave the following results. Initial amino N figures: experiment 2·38 and 2·40 cc., control 2·43 and 2·45 cc.; final amino N: experiment 1·96 and 1·95 cc., control 2·49 and 2·51 cc. The titration figures were: experiment 15·32 cc., control 19·84 cc. An experiment with alanine (0·25 g.) carried out in the same way with the quinone from 0·25 g. catechol gave initial figures of 2·45 cc. and 2·44 for the experiment and control respectively and final figures of 2·285 cc. and 2·50 cc. The titration figures were: experiment 19·30 cc., control 20·00 cc.

The control solution containing catechol in these experiments coloured slightly to a faint pink during the 24 hours' aeration but it did not produce any deamination, consequently the assumption is justified that the observed deamination in the presence of catechol and *o*-quinone was due to the latter.

Summary and Conclusions.

1. No evidence of aldehyde formation, liberation of ammonia or a diminution in amino nitrogen has been found when potato tyrosinase acts upon glycine, alanine or phenylaminoacetic acid.

2. When tyrosinase acts upon tyrosine to form melanin or its precursors oxygen is absorbed, but no oxygen absorption occurs when equivalent molecular solutions of glycine and alanine are exposed to the action of the enzyme.

3. The ammonia formation observed by Chodat and Schweizer when tyrosinase acts upon amino acids is due to the *p*-cresol which was added to the reacting system. Phenol and catechol exert the same action but not resorcinol, quinol or *p*-benzoquinone.

4. *o*-Benzoquinone alone is capable of causing deamination of amino acids in the same way as the system *p*-cresol-tyrosinase.

5. It is suggested that the deamination which takes place when tyrosinase acts upon certain phenols in the presence of amino acids is due to the preliminary formation of an *ortho*-quinone derivative which then attacks the amino acid, with the liberation of ammonia and the formation of deeply pigmented substances (Chodat's *p*-cresol-tyrosinase reaction).

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XV. THE EFFECTS OF CALCIUM GLYCEROPHOSPHATE, SODIUM GLYCEROPHOSPHATE AND SODIUM DIHYDROGEN PHOSPHATE UPON THE SKELETON OF RATS KEPT ON A DIET DEFICIENT ONLY IN FAT-SOLUBLE FACTOR.

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I. Historical.

Mellanby [1920, 1921], Korenchevsky [1921, 1922], Goldblatt [1923], Zilva, Golding and Drummond [1924] have shown that a diet deficient in fat-soluble factor produces rickets in puppies, rats and pigs. An increase in the calcium content of such a diet does not cure this type of rickets [Korenchevsky, 1923].

It is also known that human rickets cannot be cured by an increased ingestion of calcium salts. In Orgler's opinion [1911] the capacity to deposit calcium in the bones is lost during the active stage of this disease.

Grosser [1920], however, found that marked calcium retention occurred in rickety children when this element was introduced subcutaneously in the form of a glycerophosphate. As far as we know, Grosser's observations remain unique and this question has not been studied clinically or experimentally since. However, they are not only theoretically important but may be of practical use in the treatment of diseases affecting the calcium metabolism.

II. Technique of the present investigations.

We conducted 19 experiments on 20 litters containing altogether 148 rats. Clearly, for the proposed experiments, only a diet containing an adequate amount of calcium and phosphorus could be used. Therefore, during the experiment, our animals were kept on a rickets-producing diet deficient only in fat-soluble factor, because other similar diets are either deficient in calcium or phosphorus. In order to discover whether the compound calcium glycerophosphate or the glycerophosphate radical alone is the influencing factor, we divided each of our litters into two or more of the following groups.

Group I. Control group of rats kept on a diet deficient in fat-soluble factor.

Group II. Rats on the same deficient diet, a solution of calcium glycerophosphate being injected subcutaneously.

Group III. Rats on the same diet, a solution of sodium glycerophosphate being injected subcutaneously.

Group IV. Rats on the same diet, a solution of sodium dihydrogen phosphate being injected subcutaneously.

Group V. Rats on the same diet, but to which has been added calcium glycerophosphate (to show the influence of the ingestion of calcium glycerophosphate).

Two diets, $-A$ and $-A_2$, were used, both being deficient only in the fat-soluble factor; the composition of these diets is given in our previous papers [Korenchevsky, 1923; Korenchevsky and Carr, 1924]. These diets contained an abundant amount of phosphorus, *i.e.* 0·51 % in the fresh paste, and differed only in their calcium content. $-A$ diet contained an adequate amount of calcium (0·26 % in fresh paste), $-A_2$ diet an excessive amount of calcium (0·52 % in fresh paste). These different diets were employed to ascertain if an excessive ingestion of calcium might not affect the results obtained.

In two experiments, Nos. 750 and 751 (Table I), the amount of calcium in the $-A$ diet was increased, not by the addition of $CaCO_3$ as was done in $-A_2$ diet, but by the addition of calcium glycerophosphate, so that the fresh paste contained about 1·6 % of this salt. This diet contained 0·47 % Ca and 0·68 % P in the fresh paste.

The technique of the experiments was, on the whole, similar to that used in our previous investigations. The control rats always belonged to the same litter as those on glycerophosphate salts. We had great difficulty to choose a suitable preparation of calcium glycerophosphate. Grosser used Kahlbaum's salt which was soluble to about 5–6 %. But the salts now prepared by this firm as well as by English firms are only about 2·5 % soluble and are of different texture, and, when injected into rats, very often produce indurations followed by ulcerations. We were fortunate enough to obtain some of Kahlbaum's pre-war salt, which was 6 % soluble in water. This specimen of calcium glycerophosphate when injected in a 3 % watery solution was usually supported well by the rats.

As calcium glycerophosphate is more soluble in cold than in hot water it is difficult to sterilise a solution of it. Moreover we found that when dissolved in cold water, it becomes partly precipitated at 37·5° after about 24 hours from a 4 % solution and from a 5 % solution after about 4–5 hours. From this it is to be presumed that some precipitation probably occurs when such concentrations are injected subcutaneously. This probably explains the cause of the indurations and ulcerations produced, especially easily in young and weak rats on deficient diets, when 4 and 5 % solutions of this salt are injected subcutaneously. Rats on a normal diet or those put on deficient diets at an older age support injections of 4 % solutions of Kahlbaum's calcium glycerophosphate much better.

In our experiments a 3 % solution warmed to about 37° proved the most suitable. 1–2 cc. of this solution, according to the size of the animal, were injected slowly into the subcutaneous tissue four to five times a week, the place of injection being altered each time. Without all these precautions indurations and necrosis were produced at the places of injection. In some of our first experiments on larger animals we began by injecting 3·5–4 % solutions which we finally replaced by 3 %. Kahlbaum's calcium glycerophosphate which we used contained 14·72 % Ca and 11·34 % P.

We also used Kahlbaum's sodium glycerophosphate which contained 10·006 % P. A solution was prepared for injection, 1 cc. of which contained the same amount of phosphorus as did 1 cc. of the calcium glycerophosphate solution used for corresponding animals of the same litter. For example, when animals from one group of the litter were injected with a 3 % solution of calcium glycerophosphate, others from the same litter were injected with a 3·4 % solution of the sodium salt (in the experiments Nos. 833, 845, 1163, 1007, 1041). As sodium glycerophosphate may possibly be excreted by the body more rapidly than the calcium salt, in experiment No. 1204 (Table II) the rats were injected twice daily with a 6 % solution of sodium glycerophosphate. In this way we tried to keep the concentration of the sodium salt in the body as high as possible. The strength of the sodium dihydrogen phosphate (NaH_2PO_4) solution was determined on the same principle as that of the sodium glycerophosphate solution. In experiments Nos. 1226 and 1233, 2·35 % solutions of the sodium phosphate and 6 % of sodium glycerophosphate were injected once daily. Neither of these sodium salts produced any harmful local or general effects upon the rats injected.

Details of the experiments and of the results obtained are clear from Tables I–III. The theoretical calcium requirement during the period of injection, viz. 1 % of the gain in weight during that time, based on a method mentioned elsewhere [Korenchevsky, 1922], was calculated in some experiments so as to show what proportion of the calcium required normally for complete calcification of the skeleton was provided in the form of calcium glycerophosphate.

III. Ingestion of Calcium Glycerophosphate.

The influence of the ingestion of calcium glycerophosphate was studied in two small litters each containing two control rats fed on – A diet and two fed on this same diet with the addition of calcium glycerophosphate (see Table I). No difference was observed between the chemical composition of the skeleton of the animals in these two groups; the ingestion of a nearly doubled amount of calcium in the form of calcium glycerophosphate did not influence calcification at all.

The calcium ingested by the rats on – A diet and more especially by those on the calcium glycerophosphate diet was well in excess of the theoretical requirement. The average total consumption of calcium by a rat on the calcium glycerophosphate diet from litter 750 was 2·5 g. and from litter 751 was 2·4 g.

The ratio of Ca : P (1 : 1·45) in the calcium glycerophosphate diet was a good one for normal calcium metabolism.

Table I. *The influence of the ingestion of calcium glycerophosphate upon the growth and skeleton of rats kept on – A diet.*

No. of experiment	No. of rats	Duration of administration of Ca G.P. in days	Total amount of Ca administered as Ca G.P. g.	Final average weight of rats in g.		In bones			Approximate theoretical calcium requirement in g.
				Males	Females	H_2O %	Fresh Ca %	Dry Ca %	
750 controls	2	—	—	102	94	49·9	8·4	16·8	—
,, Ca G.P.	2	46	1·175	116	72	49·6	8·4	16·6	0·48
751 controls	2	—	—	126	97	46·7	8·9	16·8	—
,, Ca G.P.	2	39	1·071	105	98	48·3	8·8	16·9	0·47
Normal rats of the same age:									
—	—	—	—	—	—	43·8	11·5	20·5	—

Note. In this and the other tables the following abbreviations are used:

Ca G.P.	Calcium glycerophosphate.
Na G.P.	Sodium glycerophosphate.
Na P.	Sodium dihydrogen phosphate (NaH_2PO_4).

Therefore (1) this experiment confirms Korenchevsky's [1923] previous conclusions that the ingestion of increased quantities of calcium does not improve the rachitic calcium impoverishment of the skeleton of rats on a diet deficient in fat-soluble factor, provided that this diet already contains an adequate amount of calcium.

(2) This experiment also shows that calcium glycerophosphate is of no more value than other calcium salts for calcification of the skeleton when taken *per os*.

IV. Injection of Calcium Glycerophosphate into Rats kept on – A diet.

This series consisted of five experiments conducted on 37 rats, 22 of which were injected and 15 were controls (see Table II).

The experiment was started before the growth of the animals was arrested, because otherwise it was difficult to keep the animals alive for a sufficient length of time to give the necessary number of injections, although with rats on – A_2 diet this was done successfully in four cases (see below). The period of injection lasted from 1–3 months. The injections were begun when the animals commenced to grow more slowly and all gained in weight during the injection period (see Table). Therefore the results obtained might be attributed more to the preventive influence of the injections than to their power of curing skeletal disorders already developed. Theoretically one would expect that if the store of fat-soluble factor was not completely exhausted the injected calcium could be retained more easily by the organism and deposited in the skeleton. However, as will be shown later, there was not any special difference between the results obtained from rats which had ceased to grow and those which continued to grow during the period of injection.

When the total amount of calcium glycerophosphate injected was small, 0·08 g. and less, no influence was produced upon the skeletons of the rats (see litter 803, Table II). But when a larger amount (total of 0·17–0·22 g.) of this salt was injected in litters 787, 800, 809 and 811, calcification of the bones of these animals was much better than that of the control animals from the same litter. Comparing the injected with the control animals there was an average increase in the former of 13·4 % in calcium content of the fresh skeleton (minimal 5·4 % in litter 809 and maximal 20·7 % in litter 811) and of 14·6 % in the dry bones (minimal 8·3 % in litter 809 and maximal 20·9 % in litter 787). The water content of the bones changed very little, the average being an increase of 1·1 %.

Table II. *The influence of injections of calcium and sodium glycerophosphate and sodium dihydrogen phosphate upon the growth and skeleton of rats kept on – A diet* (*Ca content of fresh diet* 0·26 %).

No. of the experiment and of group	No. of rats	Gain (+) or loss (–) in weight in g. during period of injections		Total amount of Ca and P injected g.		In bones			Approximate theoretical calcium requirement in g.
		Male	Female	Ca	P	H_2O %	Fresh Ca %	Dry Ca %	
803 control ...	4	+29	+ 1	—	—	45·6	8·8	16·1	—
„ Ca G.P. ...	7	+29	+ 9	0·04– 0·08	—	48·6	8·4	16·3	0·1–0·3
809 control ...	4	+33	+26	—	—	45·2	9·3	16·9	—
„ Ca G.P. ...	4	+33	+29	0·17	0·13	46·3	9·8	18·3	0·3
787 control ...	2	+23	+19	—	—	45·1	8·2	14·7	—
„ Ca G.P. ...	4	+35	+11	0·18	0·14	44·9	9·8	17·8	0·25
800 control ...	3	+50	+41	—	—	39·7	11·1	18·4	—
„ Ca G.P. ...	3	+71	+50	0·20	0·15	40·7	12·0	20·2	0·6
811 control ...	2	+19	+30	—	—	41·0	8·2	14·0	—
„ Ca G.P. ...	4	+27	+19	0·22	0·16	40·6	9·9	16·7	0·23
1204 control ...	4	+24	+28	—	—	45·1	8·5	15·4	—
„ 3·4 % Na G.P.	3	+49	+27	—	0·11	45·6	8·9	16·4	—
„ 6·0 % Na G.P.	4	+45	+23	—	0·29	46·2	8·7	16·1	—
1226 control ...	2	+27	+ 7	—	—	49·8	7·4	14·7	—
„ 2·35 % Na P.	3	+12	+17	—	0·13	47·1	7·7	14·4	—
„ 6 % Na G.P.	2	+10	+ 5	—	0·13	47·4	7·7	14·7	—
		Normal calcification at the ages of litters:							
Nos. 803, 1204 and 1226	—	—	—	—	—	43·8	11·5	20·5	—
Nos. 787, 800, 809 and 811	—	—	—	—	—	36·8	14·2	22·4	—

The calcium content of the skeletons of the injected animals never reached the level found in rats of the same age on a normal diet (see Table II). This was especially clear in one litter (811) in which the amount of calcium injected was nearly as much as the total calcium requirement of the rats during this period. It is probable that some of the injected calcium glycerophosphate is excreted by the body before there has been time for the deposition of the calcium in the skeleton. *A priori*, more satisfactory results would be expected were it possible to inject larger amounts of calcium. Technically it was difficult to inject a larger amount of the salt (1) because the rats on the deficient diet become emaciated rather quickly and die and (2) because it is impossible to inject a saturated solution of the salt.

The daily injection of such large volumes of calcium glycerophosphate solution as 1·5 cc. and 2 cc. (for larger animals) did not impair the general nourishment of the rats. On the contrary, in several cases the weights of the treated animals were sometimes greater than those of the controls. From the weight figures it is difficult to draw any definite conclusion about the influence of these injections upon the growth.

V. Injection of Calcium Glycerophosphate into Rats kept on — A_2 diet.

Sixty-two rats belonging to nine litters were used for this series of experiments, 35 animals were injected and 27 remained as controls (see Table III). The duration of the injection period varied from about 1 to 1½ months, during which time 0·1–0·16 g. Ca and 0·08–0·12 g. P were injected in the form of calcium glycerophosphate solution into each animal. The results obtained were similar to and even more pronounced than those of the previous series in spite of the doubled amount of calcium in the diet. In four (869, 879, 833 and 845) of the nine litters growth practically ceased; sometimes it increased a little during the first half of the injection period and nearly always decreased during the second half. In the other five litters (1213, 1169, 1163, 1007 and 1041) the rats grew during most of the experiment, but towards the end the growth was slower and in several cases stopped. There was no essential difference between the results obtained in these two groups of litters. It is difficult to draw any conclusions concerning the effect of the injections upon the growth of the animals.

The least pronounced influence of the injections upon the skeleton was noticed in litter 1213, to each animal of which was only administered a small amount of calcium glycerophosphate containing 0·1 g. Ca and 0·08 g. P. In litter 1041 the deficient diet produced only a slight calcium impoverishment of the skeleton and the rats grew well owing to the good food given to the mother during pregnancy and lactation. One rat even became pregnant, which is unusual for animals on a diet deficient in fat-soluble factor. These facts suggest that there was a greater storage of fat-soluble factor in these animals than in those of other litters and so a fairly high calcification of the skeleton resulted in spite of the deficient diet. Because of this the injections caused the calcification of the skeleton of the treated animals to be nearly normal. The calcium content of the dry bone was normal, but the water content was greater, being 42·1 % instead of 39 %, making the calcium in the fresh bone lower than that of the normal animal (12·5 % instead of about 13·2 %).

The following figures show the average percentage changes produced by the injections in all the other litters. The calcium content of dry bone was 18·4 % (minimal 10·9 % in litter 1007 and maximal 27·7 % in litter 845) higher in the injected animals than in the controls. Similarly in fresh bone there was an increase of 16·5 % in the calcium content (minimal 7·2 % in litter 869 and maximal 27·5 % in litter 845). In most cases the water content of the bones was influenced slightly, being higher by about 2 % than that of the controls. Only in one litter, 869, did the skeletons of the injected animals contain much more water (12·1 %) than the controls.

Table III. *The influence of injections of calcium and sodium glycerophosphates and sodium dihydrogen phosphate upon the growth and skeleton of rats kept on* – A_2 *diet* (*Ca content of fresh diet* 0·52 %).

		Gain (+) or loss (−) in weight in grams during period of injections		Total amount of Ca and P injected g.		In bones		
No. of experiment and of group	No. of rats	Male	Female	Ca	P	H_2O %	Fresh Ca %	Dry Ca %
1213 control ...	2	+22	+27	—	—	48·0	8·6	16·4
,, Ca G.P. ...	3	+26	+25	0·1	0·08	50·2	9·0	18·0
869 control ...	4	0	− 5	—	—	44·6	8·3	14·9
,, Ca G.P. ...	6	− 1	− 3	0·15	0·11	50·0	8·9	17·8
879 control ...	3	−13	− 6	—	—	44·2	8·3	14·9
,, Ca G.P. ...	4	—	+ 2	0·15	0·11	46·3	9·6	17·9
1169 control ...	3	+21	+22	—	—	45·6	9·2	16·9
,, Ca G.P. ...	4	+33	+29	0·15	0·11	43·7	11·1	19·4
833 control ...	3	− 5	− 9	—	—	47·2	8·0	15·1
,, Na G.P. ...	4	− 8	− 5	—	0·11	47·4	8·4	15·9
,, Ca G.P. ...	4	0	− 6	0·15	0·11	48·7	9·1	17·8
845 control ...	3	− 4	− 9	—	—	51·5	6·9	14·1
,, Na G.P. ...	3	− 1	− 4	—	0·11	49·2	8·0	15·8
,, Ca G.P. ...	3	− 6	+ 1	0·15	0·11	51·2	8·8	18·0
1163 control ...	4	—	+12	—	—	46·6	8·7	16·2
,, Na G.P. ...	4	—	+17	—	0·11	44·6	9·8	17·6
,, Ca G.P. ...	4	+31	—	0·15	0·11	47·1	10·1	19·1
1007 control ...	2	+30	+24	—	—	45·3	10·5	19·2
,, Na G.P. ...	3	+26	+13	—	0·12	42·2	11·4	19·7
,, Ca G.P. ...	3	+28	+12	0·16	0·12	43·9	12·0	21·3
1041 control ...	3	+68	+92*	—	—	43·2	11·5	20·2
,, Na G.P. ...	3	+69	+65	—	0·12	42·9	11·5	20·4
,, Ca G.P. ...	4	+90	+49	0·16	0·12	42·1	12·5	21·5
1233 control ...	2	—	+18	—	—	47·4	7·6	14·5
,, 2·35 % Na P.	2	—	+19	—	0·13	48·5	8·0	15·4
,, 6 % Na G.P.	2	+27	+20	—	0·13	51·8	7·3	15·2
		Normal calcification at the ages of litters:						
Nos. 1213 and 869	—	—	—	—	—	43·8	11·5	20·5
Nos. 833, 845, 879, 1007, 1041, 1163 and 1169 ...	—	—	—	—	—	39·0	13·2	21·5

* This was one female rat which had become pregnant.

Table IV. *Percentage changes above* (+) *or below* (−) *the controls in the composition of the skeleton of rats injected with sodium and calcium glycerophosphates.*

	Na G.P. group In bones			Ca G.P. group In bones		
No. of litter	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
833	+0·4	+ 5·0	+ 5·3	+3·2	+13·7	+17·9
845	−4·5	+16·0	+12·1	−0·6	+27·5	+27·7
1163	−4·3	+12·0	+ 8·6	+1·1	+16·1	+17·9
1007	−6·8	+ 8·6	+ 2·6	−3·1	+14·3	+10·9
1041	−0·7	0	+ 1·0	−2·5	+ 8·7	+ 6·4
Average	−3·2	+ 8·4	+ 5·7	−0·4	+16·1	+16·2

It is interesting to note that in most cases the water content of the bones of the rats injected with calcium glycerophosphate is either the same or higher than that of the controls notwithstanding the improvement in the calcification. This shows that, although calcium glycerophosphate caused an increase in the calcium deposited in the skeleton of rats on a diet deficient in fat-soluble factor, it did not produce such a general improvement in the general composition of the bones as is caused, for instance, by the fat-soluble factor.

VI. Injection of Sodium Glycerophosphate and of Sodium Dihydrogen Phosphate into Rats kept on – A or – A_2 diets.

This series consisted of eight experiments in which 33 rats were injected. In five of these experiments, Nos. 833, 845, 1163, 1007 and 1041, the same 15 rats were used as controls as were mentioned above in Section V; each of these five litters was divided into three groups: controls, those injected with calcium, and those injected with sodium glycerophosphate (Table III). The sixth experiment, litter No. 1204, was divided into the following three groups: 4 control rats, 3 injected with 1·0 cc. of 3·4 % solution of sodium glycerophosphate once daily, and 4 injected with 1·0 cc. of 6 % solution of the same salt twice daily. This latter series of injections was made in order that the animals might retain as much sodium glycerophosphate as possible. The aim of the last two experiments, Nos. 1226 and 1233, was to compare the influence upon the calcification of the skeleton of injections of sodium glycerophosphate with those of sodium dihydrogen phosphate. Four rats were used as controls, 5 were injected with 1 cc. of 2·35 % solution of sodium dihydrogen phosphate and 4 rats with 1 cc. 6 % solution of sodium glycerophosphate once daily. Two (Nos 1204 and 1226) of these eight experiments were performed on rats kept on – A diet and the remaining six on rats kept on – A_2 diet. No difference was found in the results obtained from the injected rats on whichever of these two diets they were fed. If the influence of the injections of sodium glycerophosphate is examined in all the 28 rats treated, the following figures show the average percentage changes in the chemical composition of the bones above (+) or below (–) that of the controls.

The calcium content of dry bone was 5 % higher in the injected animals than in the controls (minimal 0 in litter 1226 and maximal + 12·1 % in litter 845). Similarly in fresh bone there was an average percentage increase of 5·5 % in the calcium content (minimal 0 in litter 1041 and maximal + 16 % in litter 845). The water content in all cases was slightly influenced, being on an average – 0·9 %. In five litters (Nos. 833, 1041, 1204, 1226 and 1233) the changes were within the usual fluctuations found in the calcification of the skeleton of rats kept on a diet deficient in fat-soluble factor.

Table IV is constructed to show the comparative value of calcium and sodium glycerophosphates in the calcification of the skeleton of rats kept on a deficient diet. In it are summarised the results obtained in those five litters mentioned above which were divided into three groups: (1) control rats, (2) those injected with calcium glycerophosphate, and (3) those injected with sodium glycerophosphate. These groups were discussed separately above. When these figures are compared (Table IV) and most particularly the average figures, it will be seen quite clearly that the injection of the calcium salt always produced a more striking effect than that of the sodium salt. The effect on calcification of the former is approximately twice as great as that of the latter. In some litters this difference is found to be even greater (litters 833, 1041, and in the calcium content of the dry bone in litter 1007). When the average percentage changes in the composition of the skeleton of all 28 rats injected with sodium glycerophosphate are compared with those of 47 rats injected with about a corresponding amount of calcium glycerophosphate the difference becomes even more striking than that in Table IV. In the first group (sodium glycerophosphate) the average percentage changes in the bones when compared with the controls were as follows: H_2O – 0·9 %, Ca in fresh bone + 5·5 % and Ca in dry bone + 5·0 %, in the second group (calcium glycerophosphate) these changes were + 0·6 %, + 15·3 % and + 17·1 % respectively.

Unfortunately, up to the present time, our experiments with sodium dihydrogen phosphate are not conclusive owing to the few rats used for the injection treatment and to the small changes and contradictory results obtained. On the basis of our previous experiences such results may only be the fluctuations usually obtained in the composition of the skeleton of rats kept on this deficient diet. In experiment 1226 (Table II) sodium phosphate did not produce any influence upon the calcification of the skeleton, whereas in experiment 1233 (Table III) the animals injected showed a slightly higher calcium content of the skeleton than the controls. In neither litter was there any appreciable difference between the effect of sodium dihydrogen phosphate and that of sodium glycerophosphate.

In a paper published simultaneously with this, Robison and Soames have found evidence in one experiment of a slight increase in the calcification of the skeleton of rats kept on – A diet after subcutaneous injections of sodium glycerophosphate, while no such increase was found after the injection of sodium phosphate [1925].

We cannot enter into a theoretical explanation of the mechanism resulting in the increase of the calcification of the skeleton after subcutaneous injections of these glycerophosphates. Robison and his co-workers [1923, 1924], who have made many interesting experiments on this subject at the Lister Institute, put forward the following theory. There is present in ossifying cartilage an enzyme which hydrolyses certain phosphoric esters including glycerophosphoric ester. By this reaction the concentration of inorganic phosphate is increased locally and calcium phosphate deposits in the bone.

VII. Histological investigation.

In all control rats kept on $-$ A or $-$ A_2 diets we found slight rickets with osteoporosis or even only the latter, because the mother rats were well fed during pregnancy and lactation. As we have mentioned several times before, it is difficult to draw any definite conclusions concerning slight histological changes observed in experimental rats, when the controls show only mild rickets and osteoporosis. In rats on $-$ A diet the histological pictures vary sometimes to a large degree principally because of more or less pronounced osteoporosis. Notwithstanding the great difference in the histological pictures, the chemical composition of the skeleton of such rats is very similar in most animals belonging to the same litter. Therefore, when only a slight influence of some agent is applied to rats on this deficient diet, the chemical results are found much more reliable and objective than the histological ones.

Bearing these limitations in mind, we may summarise the histological results of the present experiments shortly as follows. Injections of sodium phosphate and sodium glycerophosphate as well as the ingestion of calcium glycerophosphate did not alter the histological structure of the bone of the rats. When the total amount of calcium glycerophosphate injected was not smaller than 0·15 g., a definite improvement was observed in the pathological changes in the bones of about half of these animals.

Therefore, although in a lesser degree, the histological results corroborated those obtained chemically.

Conclusions.

1. The ingestion of calcium glycerophosphate, like that of other calcium salts, did not cure the rachitic calcium impoverishment of the skeleton found in rats kept on a diet deficient in fat-soluble factor only.

2. Subcutaneous injections of a watery solution of calcium glycerophosphate considerably increased the degree of calcification of the skeleton of rats kept on the same deficient diet.

3. The water content of the bones of the rats injected with calcium glycerophosphate was the same as, or higher than, that of the controls in spite of the improvement in the calcification.

4. Similar injections of sodium glycerophosphate produced in most cases the same effect as the calcium salt, but in a much less degree.

5. In about 50 % of the rats injected with calcium glycerophosphate a slight improvement of the rachitic changes in the skeleton was found histologically, which was not observed in animals injected with sodium glycerophosphate.

6. Therefore, the presence of calcium in the compound calcium glycerophosphate injected into rats on a diet deficient in fat-soluble factor seems to be of considerable importance for the greater deposition of calcium in the skeleton.

7. Calcium glycerophosphate may probably be considered as a weak therapeutical agent in the treatment of rickets produced by a deficiency of fat-soluble factor, as compared with cod-liver oil, after the ingestion of which the composition and structure of the rachitic bone of such animals returns to the normal.

8. These facts suggest a difference in the mechanisms of calcification induced in the skeleton of rats on a diet deficient in fat-soluble factor by administration of calcium glycerophosphate and of cod-liver oil respectively.

9. Our results agree with those of Grosser, who showed some retention of calcium in rachitic children after subcutaneous injections of calcium glycerophosphate.

10. No change or only a small increase in the calcification of the skeleton compared with that of controls was found in 5 rats injected with sodium dihydrogen phosphate. This increase was within the limits of the usual fluctuations observed in rats kept on the deficient diet.

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XVI. FURTHER EXPERIMENTS ON THE INFLUENCE OF THE PARENTS' DIET UPON THE YOUNG.

III. THE INFLUENCE UPON THE YOUNG OF AN EXCESSIVE AMOUNT OF CALCIUM IN THE MOTHER'S DIET DURING PREGNANCY.

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This is an addition to our previous communication [Korenchevsky and Carr, 1924] in which we omitted to take into consideration the results of some experiments we had published elsewhere [1923, 1]. In these experiments the mothers of 18 rats, belonging to three litters, Nos. 621, 624 and 646, were fed during pregnancy on N_1 (1 mother) or D_2 (2 mothers) diets (see Table, p. 113). Both these diets contained cod-liver oil. The calcium content of the former diet was adequate, but not excessive (0·26 % in fresh diet). In the latter most of the ingredients were given separately, and some in the form of paste (in particular cod-liver oil and calcium salts). Therefore, although this diet included calcium salts as well, the latter were ingested unevenly owing to the possibility of the rats selecting components of the diet poor in calcium. Roughly estimated, the rats consumed in this diet about the same amount of calcium as they did in N_1 diet, containing 0·26 % Ca.

In this paper the results obtained with these 3 litters are compared with those described in the previous paper, obtained with litters, whose mothers were fed during pregnancy on different diets. The diet of the mothers, though differing before and during pregnancy, was the same during lactation. The mothers during lactation, and the young rats after weaning, were fed on a diet deficient in fat-soluble factor (– A). The chief characteristics of the other diets mentioned in this communication are as follows. Diet N_2 was similar to the diet N_1, but differed from the latter by containing twice the amount of calcium. Diet NB_3 contained three times the amount of calcium present in NB. In both these diets the only source of fat-soluble factor was butter. All the diets used except D_2 (see above) were given in the form of a paste, by which means the amount of each ingredient eaten could be measured accurately. The technique of the experiments and details of the diets were described in our previous communications [1923, 1; 1924].

The results obtained are set forth in the Table in average figures. They have led us to *modify to some extent the first and third conclusions in our previous paper* [1924].

There was no difference between the weight or the chemical composition of the skeleton of the NB and NB_3 groups of rats (see Table). Therefore, the addition of an excess of calcium to the normal diet (containing butter as the only source of fat-soluble factor), given to the mothers during pregnancy, did not improve the condition of the young nor diminish the severity of rachitic changes in their skeletons[1].

Table.

The influence of different diets of mothers during pregnancy upon the young. The mother's diet during lactation as well as that of the young after weaning was deficient in fat-soluble factor.

Mother's diet during pregnancy	Amount of Ca % in fresh paste	Presence (+) or absence (−) of fat-soluble factor in the diet	No. of rats used for the average	Final weight of young in g.		In bones		
				♂	♀	H_2O %	Fresh Ca %	Dry Ca %
		At the day of weaning (24 *days old*).						
N_2	0·52	+(cod-liver oil and butter)	10	38	34	58·4	6·2	15·0
NB_3	0·77	+(butter only)	4	34	34	62·2	4·8	12·7
NB	0·26		63	31	30	61·4	5·0	12·7
−A	0·26	−(cottonseed oil)	5	19	19	68·3	2·6	8·2
		When 65 *days old.*						
N_2	0·52	+(cod-liver oil and butter)	25	90	85	44·5	9·3	16·6
N_1 (or D_2)	0·26		18	55	44	48·4	6·4	12·4
NB_3	0·77	+(butter only)	6	53	62	52·1	6·5	13·7
NB	0·26		62	62	53	50·9	6·8	13·8
Normal rats about 65 days old			19	140	108	43·8	11·5	20·5

However, when the mother's diet during pregnancy contained an excess of calcium *and cod-liver oil*, then the young rats showed less disorders of general nutrition and rachitic changes in the skeleton. The average figures of the weight and calcification of the skeleton of the N_1 group of rats, belonging to the litters 621, 624 and 646, and those of the N_2 group showed this quite definitely (see Table). These results emphasise the importance for the young not only of an excess of fat-soluble factor in the form of cod-liver oil, but also of ample calcium in the mother's diet during pregnancy.

It was also recorded that *at the day of birth* the Ca and P content in the young was nearly the same, irrespective of the mother's previous diet [Korenchevsky and Carr, 1923, 2]. Therefore our observations support the hypothesis, already suggested by us [1923, 1]. Our present view with regard to the influence of calcium in the mother's diet during pregnancy can be summarised as follows.

Calcium is of importance not only to the structure of the skeleton: it is a necessary component of all cells, and the nuclei contain an especially large amount of it. On the calcium content in the tissues and environment depend the working capacity and the different excitability and permeability of blood vessels, the coagulation of the blood, the resistance to certain toxins, etc. It may, therefore, be assumed that the intake of large quantities of calcium may improve the composition and functions of the mother's organism during the complex process of pregnancy, when the production of new tissues is markedly increased and the whole metabolism is altered. It is natural to expect that a more perfect organism will produce more healthy offspring, more capable of resisting deleterious pathological factors, in particular a rickets-producing − A diet.

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[1] However, when interpreting these results, it is necessary to bear in mind that butter can vary much in its fat-soluble factor content.

Probably the presence of cod-liver oil in the mother's diet during pregnancy helps to make use of the excess of calcium and favourably influences the nutrition and calcium metabolism of the young.

There are indications that feeding animals with cod-liver oil is one of the most efficient ways to increase the store of fat-soluble factor in the organism. Coward, Lush and Palmer [1923] and Goldblatt and Soames [1923] stated that the storage of growth-promoting and anti-rachitic factors in the liver of rats varies very much with the concentration of these factors in the diet. The latter authors found an increase of growth-promoting factor in the livers of those animals which were fed previously with a cod-liver oil diet as compared with those of rats fed on a butter diet.

These experiments of ours add further to the explanation, already given [Korenchevsky and Carr, 1924] with regard to the divergence of results obtained by us and by Hess and Weinstock [1924, 1]. These latter workers in their interesting investigation of the anti-rachitic effect of addition of cod-liver oil to the diet of pregnant rats used a mixed diet, which they defined "ample, although not an optimal dietary" (p. 1). Its calcium content was not estimated but seemed to be rather low. Possibly, owing to this also the results obtained by Hess and Weinstock were similar to those of ours obtained in the N_1 and D_2 groups of rats.

Here also we refer to Korenchevsky's [1922] experiments: the most severe changes of the skeleton, practically identical with rickets, were produced in young rats, which, as well as their mothers *during lactation*, were fed on a diet deficient in calcium, but containing an *ample amount of cod-liver oil.*

Hess and Weinstock [1924, 2] do not give any data with regard to the diet of the pregnant women to whom cod-liver oil was given during pregnancy with the aim of protecting their children from rickets. In their recent experiments on rats [1924, 2] cod-liver oil was not added to the diet of the pregnant animals, nor was the amount of consumed calcium determined. In their experiments the diet of the rats during pregnancy consisted of different ingredients, given separately, not in the form of a paste, which made it difficult to determine what was consumed. However, owing to the differences in experimental technique, Hess and Weinstock themselves [1924, 1, p. 6] state that "it is difficult to compare the two series of experiments" (theirs and ours). Another statement of these authors runs as follows [1924, 2, p. 1562]: "It may be stated as a principle that the resistance to rickets can be lessened by means of inadequacy of a mother's diet far more readily than a refractory condition can be brought about." Besides the results of our experiments on the influence of maternal diet upon the young, experiments of Kennedy and Dutcher [1922], Drummond, Coward and Watson [1921], Reyher [1924] and Luce [1924] showed quite definitely the great variations in growth-promoting or anti-rachitic properties of cow's milk and milk-fat, depending on the diet of the cow. Even without considering our experiments, it seems difficult to suppose that a varying amount of anti-rachitic factor in the maternal milk does not influence the resistance of the offspring to a rickets-producing diet deficient in the above factor.

Moore [1924] after giving cod-liver oil to two pregnant women observed much superior skeletal conditions in the babies born as compared with the rachitic disorders found in their previous children.

We feel that more numerous experiments are needed concerning the protective influence that the mother's feeding during pregnancy and lactation has upon the young. But we wish to emphasise once more that for these experiments and observations it is most essential to use *an optimal diet complete in every respect and to check the amount eaten*. As far as we can say at present, the presence of cod-liver oil and of an ample amount of calcium (in correct ratio with P) in such a diet is of great value.

CONCLUSIONS.

1. When the mother's "normal" diet during pregnancy was enriched with cod-liver oil and an excess of calcium, there was a marked decrease in the disorders of general nutrition and in the rachitic changes in the skeleton, produced in the young by a diet deficient in fat-soluble factor, even when the mother was also kept on the same insufficient diet during lactation.

2. This effect was not produced when the mother's "normal" diet during pregnancy contained butter as the only source of fat-soluble factor and was enriched by an excess of calcium.

3. Our experiments indicate a difference between the capability of cod-liver oil and butter to enable the animals to utilise an excess of calcium, present in the diet of the mother rats during pregnancy. At present it is impossible to state whether this difference is of a quantitative or qualitative nature owing to the large variations of anti-rachitic factor present in milk-fat.

4. Provided that the mother-rats' diet during pregnancy is complete in every respect and that the fat-soluble factor is present in the form of cod-liver oil, an ample amount of calcium in the maternal diet is of great importance for the young, kept after weaning on the above deficient diet.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Prof. C. J. Martin for his criticism of this work.

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XVII. THE PHOSPHORIC-ESTERASE OF BLOOD AT VARIOUS HYDROGEN ION CONCENTRATIONS.

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In a recent paper [Martland, Hansman and Robison, 1924] it was shown that when defibrinated blood is kept for 2–3 hours at 38° in the presence of small quantities of added acid, or in an atmosphere containing excess of CO_2, the concentration of inorganic phosphate is increased owing to enzymic hydrolysis of the phosphoric esters present in the blood. Conversely, additions of small amounts of alkali, or ventilation of the blood with CO_2-free air, gave rise under similar conditions to a synthesis of phosphoric ester with reduction in the amount of free phosphate, an observation which has been made independently by Lawaczeck [1924].

From these experiments it was suspected that the regulating mechanism which determines the activity of the esterase might prove to be the hydrogen ion concentration of the blood, but accurate estimations of p_H were not at that time made. The object of the present work was to make a closer study of this relationship, and to determine whether such changes in reaction as might be found to affect the equilibrium between the hydrolytic and synthetic activities of the enzyme are those which might occur in the blood of the living organism.

Experimental.

A method was devised by which blood at 38° might be kept at a stable p_H for a length of time sufficient for the study of the action of the phosphoric esterase. To the defibrinated whole blood of a rabbit $N/2$ sulphuric acid was added, drop by drop, in a measured amount sufficient to neutralise the plasma bicarbonate and yet insufficient for the production of acid haematin. The blood and acid were mixed and allowed to stand at room temperature for 5 minutes, to allow the hydrogen ion concentration within the corpuscles to come into equilibrium with that of the plasma, at the end of which time samples were withdrawn for estimation of p_H and of inorganic phosphate. The blood, contained in a large flask, was then kept at 38° for half an hour, during the whole of which time the flask was rotated rapidly so as to spread the blood in a thin film over the wall of the vessel, the air of the room being drawn over the blood at 5 minute intervals. By this means the CO_2 tension of the blood was brought into equilibrium with that of the surrounding atmosphere.

Samples having been withdrawn at this point for estimation of p_H and free phosphate, the blood was divided into aliquot parts and different amounts of $N/2$ caustic soda added to each. The suitable quantities of alkali had been found empirically after a considerable number of experiments, the object being to bring the p_H of the blood to values lying closely on either side of the point at which synthesis and hydrolysis are in equilibrium. The p_H and inorganic phosphate were estimated 5 minutes after adding the alkali and again after standing, without shaking, at 38° for periods varying from half-an-hour to one-and-a-half hours. No experiment was prolonged beyond two hours from the killing of the animal, it having been found that the synthetic activity of the enzyme became markedly less after that time.

Estimations of blood sugar were made parallel with the p_H and phosphate determinations, in view of the possibility that the phosphoric ester may be present in the blood in combination with a reducing group. The p_H was estimated by the method of Dale and Evans [1920], the inorganic phosphate by that of Briggs [1922], and the sugar by the method of Hagedorn and Jensen [1923].

The results of three experiments are here set forth, the phosphate and sugar being expressed as mg. per 100 cc. of defibrinated whole blood (D).

Vol. of $N/2$ H_2SO_4 per 10 cc. D	Vol. of $N/2$ NaOH added after 30 minutes cc.	Time (mins. from beginning of experiment)	p_H	Sugar mg.	Inorganic phosphate mg. P	Hydrolysis (H) or Synthesis (S) of phosphoric ester mg. P
Exp. 1.						
0·7 cc.	—	5	<6·50	125	5·15	1·48 H.
		35	6·83	128	6·63	
0·7	0·3	40	7·13	127	6·62	1·89 H.
		95	7·16	134	8·51	
0·7	0·4	40	7·36	122	6·58	1·25 S.
		95	7·38	108	5·33	
0·7	0·5	40	7·48	123	6·62	1·40 S.
		95	7·51	92	5·22	
Exp. 2.						
0·7 cc.	—	5	<6·50	128	4·92	0·79 H.
		30	6·81	128	5·71	
0·7	0·3	35	7·15	126	5·53	0·72 H.
		80	7·19	130	6·25	
0·7	0·4	35	7·35	117	5·35	0·79 S.
		80	7·39	112	4·56	
0·7	0·5	35	7·50	129	5·33	0·84 S.
		80	7·55	112	4·49	
Exp. 3.						
0·7 cc.	—	5	<6·50	137	4·88	1·20 H.
		30	6·79	141	6·08	
0·7	0·3	35	7·0	134	6·07	0·83 H.
		60	7·09	140	6·27	
		120	7·11	135	6·90	
0·7	0·4	65	7·28	136	6·65	0·31 H.
		120	7·29	133	6·96	
0·7	0·5	65	7·39	141	6·06	0·76 S.
		120	7·41	120	5·30	

These tables show a definite relationship between p_H and inorganic phosphate. The variations in the blood sugar shed no new light on previous observations. At a p_H higher than 7·3 synthesis of organic phosphate and glycolysis proceed simultaneously, but at a p_H lower than 7·3 where hydrolysis of organic phosphate takes place, the blood sugar either remains constant or shows an increase so small as to be of doubtful significance. There is no evidence of any simple and direct connection between the esterification of phosphoric acid and the synthesis of non-reducing compounds from sugar. The occurrence of glycolysis in alkalosis has been noted by various workers, and has been correlated with an increase in the lactic acid of the blood [Anrep and Cannan, 1924].

Discussion of Results.

It will be seen that after the preliminary removal of CO_2 in the first half-hour, the p_H of a given blood sample shows only very slight change from the time at which the alkali was added to the end of the experiment, and it is legitimate to assume that the reaction has remained approximately constant throughout such a period. In Exp. 3, hydrolysis of phosphoric ester has taken place at a p_H of 7·09–7·11, as also at 7·28–7·29, while at 7·39–7·41 there is decrease of inorganic phosphate indicating synthesis of the ester. The other experiments give comparable results, hydrolysis of phosphoric ester being found at every p_H lower than 7·29, and synthesis above 7·35. The critical point at which hydrolytic and synthetic processes are in equilibrium appears to lie between 7·3 and 7·35. This corresponds closely to the mean average p_H found in normal blood by various workers. Cullen and Robinson [1923] give a mean of 7·345 in normal human subjects, while in normal rabbits the mean average p_H has been found to be 7·42 for arterial blood, 7·345 for venous blood [Hawkins, 1924].

A survey of recent literature brings to light a number of instances in which alterations in the p_H of the blood *in vivo* are associated with changes in the amount of inorganic phosphate similar to those found *in vitro* in these experiments. In the observations made on a human subject by Haldane, Wigglesworth and Woodrow [1924], the acidosis induced by breathing an atmosphere containing 6–7 % CO_2 was computed to correspond to a fall of 0·09 in arterial p_H, the inorganic phosphate of the blood showing an increase from 3·39 mg. to 4·82 mg. after one-and-a-half hours under these conditions. Conversely, in alkalosis induced by forced breathing maintained for long periods, causing a reduction of alveolar CO_2 to 12 mm. and a calculated rise of about 0·3 in arterial p_H, the free phosphate of the blood was diminished to 1·25 mg. or less per 100 cc.

Kay [1924], approaching the same subject from a different point of view, has shown that the acidosis produced by ingestion of ammonium chloride gives rise to an increase of inorganic phosphate accompanied by a decrease in the amount of that fraction of the phosphoric esters upon which the esterase of the blood has been shown to act [Martland, Hansman and Robison, 1924].

The blood changes in ether anaesthesia are significant also of a close connection between increased hydrogen ion concentration and hydrolysis of organic phosphate. Van Slyke, Austin and Cullen [1922] have shown that a true acidosis occurs in this condition, and after 20 minutes' administration of ether a p_H as low as 7·07 has been recorded [Austin, Cullen, Gram and Robinson, 1924]. It has been noted that during a similar period of ether anaesthesia in rabbits the amount of inorganic phosphate is very strikingly increased [Martland and Robison, 1924].

In the terminal stages of nephritis accompanied by the so-called uraemic syndrome the association of acidosis with increase of inorganic phosphate in the blood is well established. A case is reported by Van Slyke [1921] in which Cullen, using an electrometric method, found a blood p_H as low as 6·95 in a man dying of advanced nephritis. The very marked rise in the free phosphate of the blood in such conditions has been observed by de Wesselow [1923], who attaches a grave clinical significance to this change in the phosphorus metabolism.

A relationship between acidosis and hydrolysis of organic phosphate is suggested by the work of Allan, Dickson and Markowitz [1924], who obtain a large increase of phosphates in the urine after subcutaneous injection of phloridzin in dogs. No estimations of the blood phosphates have been made in these experiments, but the amount of phosphorus eliminated is of such an order that it would be difficult to account for it without assuming an increase of inorganic phosphate in the blood. The blood p_H in phloridzinised dogs has been found by another observer to lie between 7·01 and 7·29 [Ets, 1924].

Recent work of Cavins [1924] gives rise to speculations as to whether the ketosis which occurs in carbohydrate starvation may not be concerned in a hydrolysis of phosphoric ester. In experiments on rachitic rats, Cavins has first depleted the serum of inorganic phosphate by feeding with a diet deficient in phosphate and in fat-soluble A. He has then, on fasting these animals, found an increase of free phosphate to an amount far in excess of the normal, this rise being accompanied by a cure of the rachitic condition. Control experiments on non-rachitic rats showed however no such marked hydrolysis of phosphoric ester on fasting, and it may be that factors other than the p_H of the blood have been brought into action. It would be of interest to discover whether a true "uncompensated" acidosis may not have been induced by the starvation of the rachitic animals, the alkali reserve of the normal rats having possibly been adequate to keep the blood p_H constant, or their store of glycogen having been sufficient to avert any extreme ketosis within the time-limit of the experiments.

In each of the conditions enumerated, acidosis goes hand in hand with an increase, alkalosis with a diminution, of free phosphate. It is impossible to avoid making the inference that these changes are of the same nature as those shown to occur *in vitro*, a supposition which emphasises the importance of further study of the phosphoric ester—esterase reaction of the blood.

SUMMARY.

In blood as a result of the activity of the phosphoric-esterase hydrolysis of the phosphoric ester was observed when the p_H fell below 7·3, synthesis at a p_H above 7·35.

This relationship is suggested as an explanation of certain changes in the phosphate content of the blood associated with acidosis and alkalosis, which occur in diverse conditions in the living organism.

I wish to express my thanks to Dr R. Robison for much helpful criticism and advice, and to Dr E. H. Lepper, whose experience in the detail of technique has been of invaluable aid in the determinations of blood p_H.

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XVIII. THE EARLY ACTION OF INSULIN IN THE DIABETIC.

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Even in the case of the normal animal opinion is divided as to the effect of insulin on the metabolism of carbohydrate. Macleod [1922] and others believe that the first action of insulin is to cause the storage of sugar as glycogen. Dudley and Marrian [1923], as the result of experiments on normal small animals, came to the conclusion that insulin does not cause glycogen to be stored.

The object of the present investigation was to attempt to throw some light on this subject as affecting the diabetic. It was planned to study the respiratory exchange and blood sugar levels in the diabetic as influenced by the administration of food with and without protection from insulin.

For this purpose we investigated the respiratory exchange of four severe cases of diabetes, three of whom were adult males and one a youth of 19 years of age.

That they were cases of a severe type is shown by blood sugar curves obtained from each of them after the administration of 25 grams of glucose. As judged by the trend of the curves, Case IV was the most severe, Cases I and II reacted similarly, Case III was the least severe of the series. The results obtained by the administration of glucose were on the whole confirmed by clinical examinations of the patients.

The general plan of the experiments was to administer a certain meal after a basal metabolism determination and blood sugar estimation had been made. The metabolism and blood sugar levels were followed at intervals of 15 to 30 minutes. The effect of insulin was determined by repeating the control experiment, except that 30 minutes before a given meal a dose of insulin was injected. We thus have comparative experiments which show the effect of insulin on meals of various components in cases of diabetes of varying severity. All experiments were performed in the morning, the patients having abstained from food for 12–14 hours previously. The basal metabolism determinations were made with the open circuit apparatus of C. V. Bailey [1921]. In order that the samples of air could be examined at leisure, they were collected in gas pipettes over mercury, as was done by Wolf and Hele [1914], and analysed at the close of the experiment. Over 100 basal metabolism estimations were performed on the four patients. The results are, on the whole, very uniform

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and allow us to draw some conclusions regarding the early influence of insulin on the diabetic.

Case I. The urine on admission to the hospital contained 7·6% of glucose and gave strong qualitative tests for acetone and acetoacetic acid. The experiments were commenced after a period of nine days, in which time the patient was given sugar-free and fasting regimens. With a daily diet of five eggs, ten ounces of green vegetables, and $1\frac{3}{4}$ ounces of butter, the urine contained traces of sugar, not estimable quantitatively. Acetone was present. The first experiment, after a 14 hour fast, was done with a meal containing 12·04 grams of protein, 20·0 grams of fat, with a total heat value of 238·3 Calories. The ketogenic-anti-ketogenic ratio, applying Woodyatt's formula [1922], was 2·5 : 1·0. This high ratio asserted itself in the increase in acetone compounds after the meal. From Exp. 1 it will be seen that the basal metabolism was 13 per cent. below the Dubois [1916] normal. The effect of the protein and fat was to raise the metabolism 17 per cent. within three-quarters of an hour after the meal; in two hours and a quarter the metabolism began to fall. The CO_2 was also increased, but not proportionately to the O_2 absorbed. The lung ventilation followed somewhat closely the execretion of CO_2.

The respiratory quotient in the basal state was 0·754 but with the administration of food the quotient fell, to resume its higher value at the end of the morning. The consumption of 12 g. of protein and 20 g. of fat did not materially alter the quotient.

The effect of insulin and a similar meal given to this patient is seen in Exp. 2. The experiment was performed six days after the preceding. The higher sugar concentration in the urine is explained by the fact that the patient surreptitiously obtained some bread the day before, and this was not discovered until the tests had been commenced.

The basal metabolism starts practically from the same level as in the previous experiment, namely 12 %. There did not appear to be the same influence on the metabolism due to the carbohydrate consumed the day before, as the respiratory quotient was 0·705. Lusk [1922] has recently shown that the effect of a carbohydrate meal may be traced for over 20 hours, and our findings appear to corroborate those of the American worker.

There is a small but unmistakable rise in the oxidation of carbon-rich substances, probably carbohydrate, following the injection of insulin, but the blood sugar remains constant indicating that the carbohydrate consumed was drawn from other sources than the sugar of the blood, or that a constant replacement of sugar was being effected, resulting in the glucose level in the blood being maintained. The dynamic rise in metabolism as a result of food protected by insulin is not as great as without the hormone, nor does the lung ventilation follow so closely the CO_2 elimination. There is again a definite drop in the respiratory quotient following the ingestion of food, but what is of greater interest is that metabolism is transferred almost entirely to the combustion of protein and fat, since in the triangular diagrams of

Case I. Computed oxygen absorption in the basal state: *217 cc. per min.*

		Per min.				Litres	Blood	Urine %		
	Time	O_2 c.c.	B.M.R. %	CO_2 cc.	R.Q.	Lung vent.	% sugar	sugar	acetone	Remarks
Exp. 1	9.30	—	—	—	—	—	0·223	Trace	+	Absolute rest
	10.15	187	– 13	141	0·754	5·19	—	—	—	
	10.30	—	—	—	—	—	—	—	—	P. 12 g., F. 20 g., C. 0 ingeste
	11.0	—	—	—	—	—	0·206	—	—	Patient quiet
	11.20	226	+ 4	166	0·735	5·79	—	—	—	
	12.40	215	– 1	161	0·750	5·75	—	—	—	
	12.45	—	—	—	—	—	0·203	Trace	+ + +	
Exp. 2	9.10	—	—	—	—	—	0·262	0·2	3·7	Absolute rest
	9.40	191	– 11	135	0·705	5·02	—	(g. per 1000 cc.)		
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10·25	199	– 8	149	0·749	5·40				
	10.30	—	—	—	—	—	0·260			
	10.40	—	—	—	—	—	—	—	—	P. 12 g., F. 20 g., C. 0 ingeste
	11·10	217	± 0	155	0·715	5·23	—	—	—	Patient quiet
	11.30	—	—	—	—	—	0·208	—	—	
	11·45	222	+ 2	166	0·740	5·43	—	—	—	
	12.15	219	+ 1	166	0·760	5·42	—	—	—	
	12.35	215	– 1	165	0·765	5·35	—	—	—	
	12·45	—	—	—	—	—	0·162	0·2	1·9	
Exp. 3	9.10	—	—	—	—	—	0·195	0	6·8	Absolute rest
	9.45	181	– 16	129	0·710	5·05	—	—	—	
	10.0	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g ingested
	10.45	223	+ 3	157	0·705	5·63	—	—	—	Patient quiet
	11.0	—	—	—	—	—	0·187	—	—	
	11.15	233	+ 7	171	0·735	6·20	—	—	—	
	11.40	241	+ 11	182	0·755	6·37	—	—	—	
	12.20	232	+ 7	183	0·790	6·38	—	—	—	Becoming a little restless
	12.30	—	—	—	—	—	0·270	1·58	7·5	
Exp. 4	9.10	—	—	—	—	—	0·165	0·37	1·06	Absolute rest
	9.45	171	– 21	121	0·710	5·09	—	—	—	
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10.25	189	– 13	139	0·735	5·20	—			
	10.30	—	—	—	—	—	0·285	—	—	P. 16·4 g., F. 24·7 g., C. 27 g ingested
	11.15	225	+ 3	160	0·710	5·54				
	11.30	—	—	—	—	—	0·221			
	11.45	220	+ 1	173	0·785	5·31				
	12.10	219	+ 1	172	0·783	5·37				
	12.30	212	– 2	160	0·755	5·32				
	12.45	—	—	—	—	—	0·247	0·43	0·87	
Exp. 5	9.10	—	—	—	—	—	0·115	0	0·096	Absolute rest
	9.40	173	– 20	125	0·721	4·93	—	(g. per 1000 cc.)		
	9.50	—	—	—	—	—	—	—	—	*Insulin* U. 15
	10.15	189	– 13	143	0·755	5·18				
	10.20	—	—	—	—	—	0·100			
	10.30	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g ingested
	11.10	214	– 1	144	0·673	5·39	—	—	—	Patient quiet
	11.25	—	—	—	—	—	0·141	—	—	
	11.40	232	+ 7	165	0·711	5·61	—	—	—	
	12.5	231	+ 7	167	0·723	5·67	—	—	—	
	12.35	226	+ 4	165	0·732	5·64	—	—	—	
	12.40	—	—	—	—	—	0·146	0	0·119	

Case I (*continued*)

	Time	Per min. O_2 cc.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres Lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
p. 6	9.10	—	—	—	—	—	0·187	0	0	Absolute rest
	9.45	198	− 8	144	0·730	5·23	—	—	—	
	9.55	—	—	—	—	—	—	—	—	*Insulin* U. 20
	10.20	190	−12	147	0·776	5·57				
	10.25	—	—	—	—	—	0·171			
	10.30	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40·5 g., ingested
	11.0	218	+ 1	159	0·730	5·65	—	—	—	Patient quiet
	11.5	—	—	—	—	—	0·265	—	—	
	11.30	212	− 2	167	0·788	5·60	—	—	—	
	12.0	213	− 2	164	0·772	5·55	—	—	—	
	12.40	201	− 7	161	0·800	5·63	—	—	—	
	12.45	—	—	—	—	—	0·216	0	0	
p. 7	9.10	—	—	—	—	—	0·156	0	Trace	*Computed* B.M.R. 223 cc. O_2
	10.10	229	+ 3	165	0·723	6·57				
	10.15	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 g., ingested
	10.55	—	—	—	—	—	0·174	—	—	Patient quiet
	11.0	220	− 2	150	0·685	5·52	—	—	—	
	11.30	247	+10	188	0·763	6·41	—	—	—	
	12.0	244	+ 9	206	0·846	6·39	—	—	—	
	12.30	239	+ 7	186	0·780	6·52	—	—	—	
	12.45	—	—	—	—	—	0·266	1·2	Trace	

Case II. Computed oxygen absorption in the basal state: *204 cc. per min.*

	Time	Per min. O_2 cc.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
xp. 10	9.20	—	—	—	—	—	0·090	0	Trace	Absolute rest
	10.5	186	− 9	135	0·726	5·74	—	—	—	
	10.20	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.0	210	+ 3	147	0·701	6·02	—	—	—	Patient quiet
	—	—	—	—	—	—	0·130	—	—	
	11.40	214	+ 5	159	0·743	6·77	—	—	—	
	12.15	213	+ 4	165	0·777	6·70	—	—	—	
	12.50	218	+ 7	165	0·756	6·63	—	—	—	
	1.0	—	—	—	—	—	0·232	Trace	Trace	
xp. 11	9.10	—	—	—	—	—	0·114	0	0	Absolute rest
	9.45	187	− 8	134	0·715	5·51	—	—	—	
	9.50	—	—	—	—	—	—	—	—	*Insulin* U. 15
	10.15	174	−14	128	0·732	5·27				
	10.25	—	—	—	—	—	0·106			
	10.35	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.15	201	− 1	144	0·714	5·48	—	—	—	Patient quiet
	11.20	—	—	—	—	—	0·115	—	—	
	11.40	199	− 2	147	0·738	5·53	—	—	—	
	12.10	202	− 1	156	0·775	5·59	—	—	—	
	12.40	202	− 1	157	0·779	6·01	—	—	—	
	12.45	—	—	—	—	—	0·116	0	0	

Dubois the oxidation of carbohydrate takes very little part in a metabolism the respiratory quotient of which is 0·715. It will be noticed, however, that the respiratory quotient rose steadily, the final figure being above that found in the companion experiment without insulin.

Disregarding any calculations as to the relationships between the blood sugar levels and the respiratory quotient, one may only say that the acetone concentration in the urine was less at the end of the experiment, indicating a more efficient combustion of fat, possibly due to the utilisation of carbohydrate as the result of the use of insulin.

Comparing the results obtained in the two experiments, it will be seen that the immediate effect of insulin is to increase the combustion of carbohydrate in the post absorptive state. Following the ingestion of the diet, the combustion is apparently transferred to the combustion of protein and fat, since the respiratory quotient falls at first, reaching a higher level at the end of 2½ hours when assisted by insulin than without it. What also is noteworthy is that the total metabolism following the ingestion of food and insulin is lower than without the hormone.

The next three experiments are concerned with the metabolism of the diabetic consuming meals containing protein, fat and carbohydrate. The effect of varying the amounts of insulin injected was investigated. The protocols are given under Expts. 3, 4 and 5. The meal contained protein 16·4 grams, fat 24·7 grams, carbohydrates 27·0 grams. Total calories 408. Ketogenic-anti-ketogenic ratio 0·75 : 1. Exp. 3 shows the simple experiment where no insulin was administered. That the patient was incapable of catabolising the available carbohydrate in the meal is shown by the fact that while the urine was sugar-free at the commencement of the experiment, the concentration of glucose at the end was 1·58 %. The effect of the meal containing all three principal food components is to raise the total metabolism 7 % above the level obtained with fat and protein alone. That this increase does not result from an increased combustion of the carbohydrate is shown by the fact that the rise in the total metabolism is not coincident with the highest respiratory quotient. One may therefore conclude that it is due to the promoting action of the carbohydrate on the combustion of protein and fat. The rise in the blood sugar level does not take place until the final specimen, when a value, of 0·27 % was obtained. This corresponds to the finding of sugar in the urine of 1·58 %. In spite of the ketogenic-anti-ketogenic ratio of the food being 0·75 : 1·0, the excretion of acetone bodies was increased after food, due doubtless to the inefficiency of carbohydrate utilisation.

Exp. 4 was done six days later and demonstrates the effect of 10 units of insulin upon the metabolism after the ingestion of protein, fat and carbohydrate. The basal metabolism on this morning was—21·0 % with a respiratory quotient of 0·71. There was a trace of sugar in the urine in spite of the fact that the blood sugar was 0·17 %.

The immediate effect of the injection of 10 units of insulin was to increase the combustion of carbohydrate as shown by the elevation of the respiratory quotient from 0·71 to 0·735. The drop in the respiratory quotient as the result of giving food is well seen and the rise to a value of 0·785 is thereafter rapid. As the lowest respiratory quotient is coincident with the greatest oxygen absorption it is evident that carbohydrate can have taken little part in the elevation of the metabolism.

The amount of insulin administered was not sufficient to protect the patient from a characteristic rise in blood sugar, which reaches its highest point in the final blood sugar estimation $2\frac{1}{2}$ hours after the patient received food. Whether insulin has an effect on the absorption of carbohydrate from the alimentary canal, which seems unlikely, or whether some step in the intermediary fate of the food stuff is accelerated, there appears to be a more immediate rise in the level of blood sugar with than without insulin, and this phenomenon we have seen in others of our experiments. As the commonly accepted ratio between the amount of sugar capable of being catabolised per unit of insulin used is as 2 : 1 and the food contained in all 39 g. of available carbohydrate from the bread, 9·52 from the protein, and 2·47 from the fat, the rise in blood sugar and increase in urinary sugar was to be anticipated with only 10 units of insulin given.

In order to see if an increase in insulin dosage would alter the type of curve, another experiment was performed seven days later (Expt. 5). The daily diet had been increased until the patient was receiving 1153 Calories per day, of which 56 Calories were contributed by carbohydrates. The same meal as in the previous experiment was administered, preceded, however, by 15 units of insulin. In this experiment the amount of insulin was sufficient to keep the blood sugar below the threshold for this case and there was, therefore, no sugar excreted in the urine. As will be seen, the trend of the total respiratory exchange and respiratory quotients is similar to that in the previous experiment, the respiratory quotient falling as the result of the administration of food. There is no late depression in total metabolism. Viewing this experiment as a whole it seems that the organism was able to utilise the greater part of 39 g. of carbohydrate. That the low respiratory quotients were not due to a retention of CO_2 by the liberated alkali on the disappearance of the ketonic acids from the blood, as Davies and his co-workers suggest, is negatived by the fact that the concentrations of acetone compounds in the urine at the beginning and end of the experiments are not in harmony with this idea. If large quantities of lactic acid were formed, there would be a tendency to an increased lung ventilation. There was, as a matter of fact, a decrease. The only theory therefore which appears to satisfy the condition is a transformation of the carbohydrate into glycogen or into a substance of unknown composition.

At this point in his treatment the patient received 15 units of insulin daily, administered in three 5-unit doses. His total consumption of food, 57·3 g. as protein and 37·6 g. as carbohydrate, was equivalent in all to 1471 Calories. After seven days of this treatment we estimated the effect of three ounces of bread in one meal after protection by 20 units of insulin given in one dose (Exps. 6 and 7). As the bread given contained 40·5 g. of carbohydrate, 6·6 g. of protein, and 6·6 g. of fat, the total amount of available glucogenic material was 44·0 g. Assuming the customary ratio between carbohydrate and insulin we should have expected practically complete protection by the hormone. The results of giving the three ounces of bread preceded by an injection of 20 units of insulin were as follows.

Owing, doubtless, to his continued insulin treatment, the basal metabolism on this day was—8·0 %, 12 % higher than that obtained in the previous week. The respiratory quotient was 0·730, indicating a metabolism concerned chiefly with the combustion of protein and fat. The diet even with the amount of insulin given contained an excess of carbohydrate, as shown by the high blood sugar, and the presence of glucose in the urine. After the 14 hour fast the urine was sugar- and acetone-free.

The immediate effect of 20 units of insulin was to depress the total metabolism and elevate the respiratory quotient. This immediate depression of the total metabolism was not seen in the previous experiment with smaller dosages of insulin, but was seen in other of our experiments, notably case II, where 10 units were sufficient to depress the total metabolism. It is not altogether easy to explain this depression. Kellaway and Hughes suggest the transformation of carbohydrate to some substance unknown, the change involving an economy of oxygen and a liberation of excess of carbon dioxide. It is possibly also a protective phenomenon in which the proportion of carbohydrate burnt is increased and thus spares protein and fat. This involves a lower amount of oxygen used and hence is represented in indirect calorimetry as a fall in the total metabolism. It will be noted that during this period the respiratory quotient rose.

The ingestion of three ounces of bread raised the metabolism 9 % above the determined basal level, whereas protein and fat of the same caloric value decreased the level 17 %. The blood sugar rose almost immediately after the ingestion of bread to 0·265 %. With this figure the respiratory quotient rose in the course of the morning to 0·80, the highest obtained with the patient.

Twenty units of insulin in this patient depress the total metabolism and increase the proportion of carbohydrate utilised. The drop in the respiratory quotient after food is again pronounced and does not confirm the observation of Wilder, Boothby and Beeler [1922], who state that the depressor effect is greatest after protein.

Two days after the foregoing series of tests was performed, the daily dose of insulin was increased to 24 units. With this three ounces of bread were given, one after each injection. Sugar was still present in the urine. The dose of insulin was raised to 30 units per day, and finally to 45 units per day. With this dose the patient remained aglycosuric and added 11 pounds to his weight. The effect of giving three ounces of bread without the protection of insulin was now determined. It will be noted (Exp. 7) that the basal metabolism had risen as the result of treatment from − 20 % to + 3 %, confirming Joslin's observations [1918] and lending support to the contention that the basal metabolism is really low in severe cases of diabetes.

In the present experiment the specific dynamic action of the carbohydrate followed on the rise in the respiratory quotient and indeed the total metabolism had already fallen when the respiratory quotient was still on the increase.

There does not seem to have been any increase in carbohydrate tolerance, for, referring to Exp. 3, it will be seen that 39 g. of available carbohydrate raised the blood sugar at the end of 2½ hours to 0·270 %, while in the present experiment, the blood sugar rose to 0·266 % after three ounces of bread. There was undoubtedly a certain amount of carbohydrate utilised, as the respiratory quotient of 0·846 indicated, but the 20 units of insulin given did not produce a normal catabolism of sugar.

Having discussed in some detail the results obtained with one case of diabetes we now present the results of experiments with three others. These results will be discussed only in so far as they bear comparatively on the first case.

Case II, aged 50, was one with a marked family history of diabetes. The urine on admission contained 6·9 % of glucose; the blood sugar was 0·245 %. He reacted very quickly to fasting, the urine becoming sugar free in two days. Exps. 10 and 11 were performed on this patient. The results go to show that insulin by itself enables the reserve carbohydrate to be catabolised, and at the same time depresses the total metabolism, and furthermore lowers the dynamic action of a given meal when injected 30 minutes prior to the meal.

Case III is presented as that of a diabetic with a history of loss of weight extending over ten years. As will be seen from Exps. 14, 15, 16 and 17, this patient reacted less promptly to insulin. The elevation of the respiratory quotient from insulin alone was present but less marked. The depression of the specific dynamic action of protein was not seen. There was little increase in carbohydrate tolerance even after treatment with insulin for 45 days, showing the refractory nature of the condition. Case IV is presented as a contrast to the foregoing. The patient was a youth of 19 years of age, who had been treated in the hospital about eight months previously, during which time his tolerance had been raised so that he was able to take Graham's full diet with two ounces of brown bread and one ounce of potatoes. His urine at that time was free from sugar and the blood sugar was 0·086 %.

On re-admission the urine contained 4·2 % of glucose. After 25 g. of glucose the blood-sugar rose to 0·340 %. As will be seen from Exp. 18, the effect of insulin on this patient was very striking. It produced within 25 minutes an elevation of the respiratory quotient from 0·763 to 0·830, with a drop of 7 % in the oxygen absorption.

The administration of two ounces of bread increased the metabolic rate 13 % above the basal value. The stimulation of metabolism was only transitory.

In two hours, however, the respiratory quotient had risen to 0·890, indicating that a considerable quantity of carbohydrate was being oxidised.

The second experiment with this patient was a control of the foregoing. He received two ounces of bread without the preliminary protection with insulin. The effect of this is strikingly shown in Exp. 19. The basal oxygen absorption had dropped to – 12 % and the respiratory quotient was at the high level of 0·877. The blood sugar level had fallen to 0·114 %, the urine was sugar-free and contained only a trace of acetone. Obviously the patient was reacting promptly to the restricted diet which was that of Graham's third day.

Case III. Computed oxygen absorption in the basal state: *210 cc. per min.*

		Per min.						Urine %		
	Time	O_2 cc.	B.M.R. %	CO_2 cc.	R.Q.	Litres lung vent.	Blood % sugar	sugar	acetone	Remarks
Exp. 14	9.15	—	—	—	—	—	0·133	0	2·19	Absolute rest
	9.55	206	– 2	144	0·699	5·59	—	—	—	
	10.20	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 ingested
	10.55	226	+ 8	151	0·667	5·20	—	—	—	Patient quiet
	11.0	—	—	—	—	—	0·191	—	—	
	11.30	226	+ 7	166	0·735	5·50	—	—	—	
	12.0	242	+15	164	0·676	5·42	—	—	—	
	12.30	247	+18	170	0·687	5·75	—	—	—	
	12.40	—	—	—	—	—	0·195	2·76	2·16	
Exp. 15	9.15	—	—	—	—	—	0·083	0	1·90	Absolute rest
	9.55	210	± 0	142	0·677	4·78	—	—	—	
	10.0		—	—	—	—	—	—	—	*Insulin* U. 15
	10.25	208	– 1	144	0·690	4·95				
	10.30	—	—	—	—	—	0·112			
	10.35	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 ingested
	11.15	235	+12	154	0·656	5·12	—	—	—	Patient quiet
	11.20	—	—	—	—	—	0·178	—	—	
	11.45	243	+15	175	0·719	5·45	—	—	—	
	12.40	248	+18	183	0·739	5·63	—	—	—	
	12.45	—	—	—	—	—	0·170	0	1·30	
Exp. 16	9.10	—	—	—	—	—	0·127	0	Trace	Absolute rest
	9.45	210	± 0	140	0·669	4·56	—	—	—	
	9.50	—	—	—	—	—	—	—	—	*Insulin* U. 20
	10.15	194	– 7	132	0·680	4·55	—			
	10.20	—	—	—	—	—	0·113			
	10.30	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 ingested
	11.0	217	+ 3	144	0·680	4·74	—	—	—	Patient quiet
	11.10	—	—	—	—	—	0·210	—	—	
	11.30	239	+14	166	0·696	5·19	—	—	—	
	12.0	232	+10	160	0·692	5·00	—	—	—	
	12.30	234	+11	158	0·674	5·01	—	—	—	
	12.45	—	—	—	—	—	0·182	1·25	Trace	
Exp. 17	9.30	—	—	—	—	—	0·110	0	0	Absolute rest
	10.0	200	– 5	145	0·723	4·70	—	—	—	
	10.15	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 ingested
	11.20	222	+ 6	166	0·749	6·18	—	—	—	Patient complained of pain application of the nose
	11.30	—	—	—	—	—	0·230	—	—	
	12.15	218	+ 4	160	0·736	5·06	—	—	—	
	12.30	—	—	—	—	—	0·232	2·34	0	

Case IV. Computed oxygen absorption in the basal state: *223 cc. per min.*

		Per min.						Urine %		
	Time	O_2 cc.	B.M.R. %	CO_2 cc.	R.Q.	Litres lung vent.	Blood % sugar	sugar	acetone	Remarks
p. 18	9.10	—	—	—	—	—	0·210	0	Trace	Absolute rest
	9.50	215	− 3	164	0·763	4·75	—	—	—	
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10.25	200	− 10	166	0·830	5·31				
	10.30	—	—	—	—	—	0·204			
	10.35	—	—	—	—	—	—	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	11.5	246	+ 10	192	0·781	5·31	—	—	—	
	11.15	—	—	—	—	—	0·246	—	—	
	11.35	221	− 1	174	0·790	5·38	—	—	—	Patient quiet
	12.0	211	− 5	164	0·777	5·21	—	—	—	
	12.30	209	− 6	186	0·890	6·19	—	—	—	
	12.45	—	—	—	—	—	0·244	0	Trace	
p. 19	9.20	—	—	—	—	—	0·114	0	Trace	Absolute rest
	10.0	195	− 12	171	0·877	6·41	—	—	—	
	10.15	—	—	—	—	—	—	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	10.55	225	+ 1	167	0·741	6·05	—	—	—	
	11.0	—	—	—	—	0·184	—	—	—	
	11.30	217	− 3	164	0·756	5·59	—	—	—	Patient quiet
	12.0	212	− 5	166	0·783	5·72	—	—	—	
	12.30	211	− 5	165	0·783	5·35	—	—	—	
	12.45	—	—	—	—	—	0·240	Trace	Trace	
p. 20	9.15	—	—	—	—	—	0·122	0	Trace	Absolute rest
	10.0	197	− 11	166	0·847	5·63	—	—	—	
	10.5	—	—	—	—	—	—	—	—	5 *pan-secretin tablets given*
	10.30	194	− 12	164	0·845	5·18	—	—	—	
	10.35	—	—	—	—	—	0·118	—	—	
	10.40	—	—	—	—	—	—	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	11.30	224	+ 1	177	0·791	5·52	—	—	—	
	12.0	198	− 11	160	0·807	5·35	—	—	—	Patient quiet
	12.45	201	− 10	158	0·788	5·84	—	—	—	
	12.50	—	—	—	—	—	0·218	Trace	Trace	

The immediate effect of the food was most striking. The respiratory quotient fell to 0·741 and the oxygen absorption was increased. The specific dynamic action of the carbohydrate was only transitory, for by the end of the morning the oxygen absorption had fallen to − 5 %. The respiratory quotient did not rise to the high figure reached in the experiment with insulin, the quotient generally indicating a restricted use of sugar. The blood sugar curve is typically diabetic, the last point reaching the value 0·24 %. These experiments strongly suggest that the immediate effect of insulin when injected into a fasting diabetic is to depress the total metabolism and at the same time to increase the proportion of sugar oxidised. Probably the former is contingent on the sparing action of the carbohydrate. This, however, is not the only action of insulin, for the final high respiratory quotient reached after the injection of 10 units of insulin and not seen in the experiment without insulin can be explained by assuming that insulin enabled the organism to oxidise a greater proportion of sugar, not, however, immediately after the ingestion of this food, but after the stimulating effect of the carbohydrate on the total metabolism had passed off. This delayed oxidation of sugar may be accounted for by assuming that the first action of insulin after the initial small combustion of sugar is to convert the carbohydrate into some intermediary complex and later to assist in its combustion.

Finally, a preliminary experiment is here reported on the effect of the oral administration of a preparation of the pancreas. The makers do not claim an antidiabetic action except after long continued administration, but it was hoped to demonstrate some effect As will be seen, Exp. 20, five "pan-secretin" tablets given in place of 10 units of insulin produced no immediate effect on the respiratory quotient, the total metabolism or the blood sugar percentage. There is no evidence of an increased utilisation of sugar due to "pan-secretin." Hence this product cannot be substituted for insulin when immediate results are to be anticipated.

DISCUSSION.

The reaction to food and insulin depends on the age and the condition of the patients. The youngest patient of the series reacted quickly to a single dose of insulin. This case showed considerable capacity for oxidising carbohydrate $2\frac{1}{2}$ hours after the injection of insulin. A long standing case of the disease as illustrated by Case III showed very little evidence of any ability to utilise carbohydrate better as the result of diet and insulin. It is notable that the basal metabolic rate of the former case increased as the result of treatment while the rate of the latter remained stationary. As the basal metabolic rate increased, as the result of treatment with diet and insulin, with no corresponding increase in weight of the patient, one may advance this fact in support of the idea that the basal metabolism is really decreased in diabetes apart from any consideration of loss of weight or decreased plane of nutrition. The tolerance of the first case showed a decided improvement during his stay in hospital, while the ability of the second case to utilise carbohydrate was not increased.

The presence and function of a small store of reserve carbohydrate is made evident in these experiments. It is to this store that the metabolism is directed by the injection of insulin. Immediately the reserve carbohydrate commences to be catabolised the protective action on the protein metabolism is manifested and the total respiratory exchange falls, more or less coincidently with a rise in the respiratory quotient.

Once the metabolism has been directed into more normal channels, the organism appears to attempt to preserve the store of carbohydrate and the protein and fat are now attacked. This is the second phase, evidenced by the return of the respiratory quotient to a lower level. In some instances this is paralleled by an increase in the total respiratory exchange.

The experiments do not bear out the theory of Davies, Lambie *et al.* [1923] that the delayed elevation of the respiratory quotient is due to a retention of carbon dioxide by the liberated alkali on the disappearance of the keto-acids from the blood, for the curves of acetone excretion in the urine do not correspond with this. Nor do the experiments support the theory of Briggs and Koechig [1924], viz. that the action of insulin is to promote the conversion of glucose into lactic acid, for there is no substantial evidence of an increased lung ventilation, which we would anticipate if such were the case.

The observations seem to fit in rather with the more obvious explanation that the first action of insulin is to convert glucose into glycogen, and as soon as a reasonable demand of the tissue for this substance is satisfied, the insulin enables the organism to mobilise and burn the glycogen in the normal way.

The fact that Dudley and Marrian were unable to demonstrate an increased storage of glycogen in the livers and muscles of normal animals after treatment with insulin does not appear to disprove the theory that the first action of insulin is to convert glucose into glycogen or a similar substance and later to assist in its combustion, but rather tends to confirm it. In the normal animal there is already a sufficiency of glycogen in the tissues. The action of insulin in the normal would be to bring about a combustion of this glycogen rather than a polymerisation and storage of glucose. Hence the experiments on the normal animal only apply with reserve to the processes taking place in the diabetic.

We wish to tender our thanks to Dr E. Lloyd Jones and to Dr Aldren Wright for permitting us to investigate the cases in their service at Addenbrooke's Hospital; to the Sister of Griffith Ward for her unfailing assistance, and to the Medical Research Council for a grant to one of us, part of which has been used in the present work.

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XIX. THE INFLUENCE OF OXYGEN ON THE PRODUCTION OF UREA BY ENZYMES OF THE LIVER AND SPLEEN.

By ROBERT ALEXANDER McCANCE.

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(*Received November 11th, 1924.*)

In a previous paper, McCance [1924], the production of urea by mammalian spleen, liver and kidney in autolysis was described, and mention made of the marked inhibition of this reaction by performing the autolysis in pure oxygen gas. This inhibition has now been further studied. The general methods used to estimate the urea and regulate the p_H were described in the last paper and need no further comment.

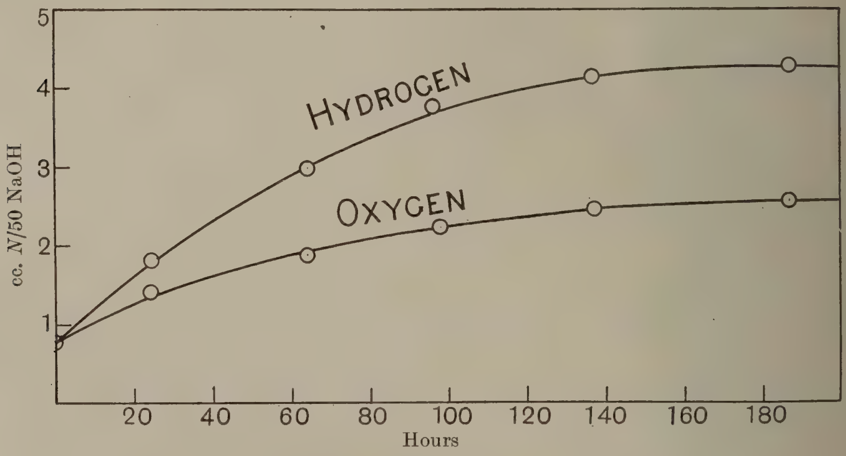
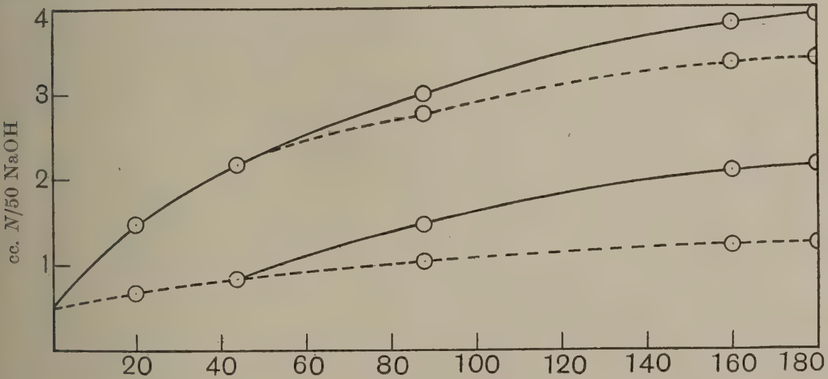


Fig. 1.

Fig. 1 shows the effect of oxygen on the production of urea by a spleen (ox) autolysing at p_H 6. The experiment was arranged as follows. Spleen was made up for autolysis at p_H 6, sampled, halved, placed in two filter flasks, and evacuated till free from air. One flask was then filled with oxygen, and one with hydrogen, well shaken, and both incubated at 37°. Each flask was evacuated and refilled with gas after sampling. Amounts of urea are expressed in cc. $N/50$ NaOH. Results such as this may always be obtained, the two curves ultimately becoming parallel to each other and to the time axis, showing that the total amount of urea produced is always much less in oxygen than in hydrogen.

The previous paper showed that the effect of oxygen gas was upon the enzyme, not upon the substrate. The following experiment (Fig. 2), which is one of several carried out, shows that while this oxygen effect is (largely at any rate) reversible, an autolysis begun in oxygen, even if subsequently changed to hydrogen, still shows a much smaller total production of urea than if kept in hydrogen gas from the beginning. The full curves indicate urea production in hydrogen, the dotted curves in oxygen. Autolyses of spleen at p_H 6 were set up exactly as described above in oxygen and hydrogen gas, and samples taken after one, and after two days. Each was then halved, and half the autolysis in oxygen was transferred after rapid and complete evacuation to hydrogen. In a similar way half the autolysis in hydrogen was transferred to oxygen. After the change over the two autolyses in hydrogen (one has been in hydrogen throughout, the other has been changed from oxygen to hydrogen) both show roughly parallel rates of production of urea. Thus, although the *activity* of the enzymes producing urea has not been much, if at all, reduced by the treatment with oxygen, yet the total *amount* of urea produced by enzymes so treated is always less than that of their fellows which have been kept in hydrogen throughout; and this although oxygen does not destroy the substrate.



Hours

Fig. 2.

If the observations of the effect of oxygen gas on the production of urea are extended to autolysing liver, the results are different. At p_H 5 (where there is no formation of urea whatever in the spleen) the inhibition is slight but of essentially the same nature as that shown in Fig. 1, whereas at p_H 6 it may be, and usually is, completely absent, though there is a formation of urea.

Of quite a different nature are the curves in Fig. 3 showing the effect of oxygen on the production of urea by liver enzymes from the filtrate of a spleen completely autolysed at p_H 5. (As described in the previous paper, there is no formation of urea during the complete autolysis of spleen at p_H 5, but a very complete hydrolysis.) The procedure by which the curves were obtained was as follows: 30 cc. of the autolysate, previously boiled, filtered, and titrated to p_H 7, were placed in each of two small filter flasks, evacuated, and filled, one with oxygen, the other with hydrogen. To prepare the enzyme suspensions, liver was made up as for autolysis at p_H 6, strained through muslin, and treated with oxygen and hydrogen exactly as described above in the case of the spleen. These remained 24 hours at room temperature, and were then incubated 14 hours at 37°. 3 cc. were then withdrawn from the suspension in hydrogen and added to the 30 cc. autolysate in hydrogen. Similarly 3 cc. from the suspension in oxygen were added to the autolysate in oxygen. To sample, 5 cc. were withdrawn at once, and again at suitable intervals, placed in boiling water, and estimated. After sampling, each flask was re-evacuated and refilled with gas. There is no formation of ammonia under these circumstances. The essential difference between the effect of oxygen gas as illustrated by Figs. 1 and 3 is that in Fig. 1 the oxygen gas reduces the rate of formation of urea and also the total amount formed, whereas in Fig. 3 the effect is only upon the rate, the total amount formed being ultimately the same in both oxygen and hydrogen. Fig. 3 completely bears out the conclusion, reached previously by a different method, that the oxygen does not exert an irreversible effect on the substrate.

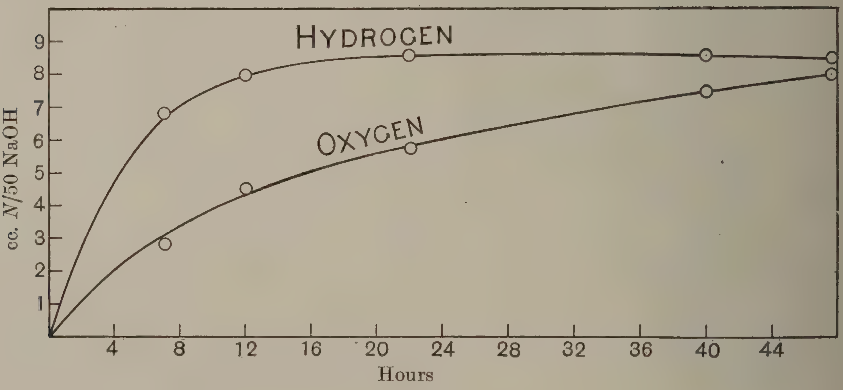


Fig. 3.

Only one explanation remains therefore of why the *total amount* of urea produced in an autolysis (Fig. 1) is less in oxygen than in hydrogen, although the effect of oxygen is only upon the velocity of urea production, and is reversible. The reactions giving rise to urea in such an autolysis come to an end, because the enzymes, whether in oxygen or hydrogen and apart from the influence of either gas, become inactive. This occurs before the enzymes treated with oxygen have time to decompose as much of their substrate as the enzymes treated with hydrogen. Two separate factors leading to this result have been discovered.

(1) The production of urea is greatly inhibited by the products of autolysis. While fractionating the products of a complete autolysis of spleen at p_H 5,

the whole of the precursor was found to be removed from solution by silver in saturated baryta solution, and it was noticed in studying the urea formation from this basic fraction how much more active the enzyme appeared to have become. The same effect was observed after a butyl alcohol extraction. The full curves (Fig. 4) show this inhibition due to products of autolysis. The dotted curve beside each full curve shows the inhibition due to oxygen (which is very slight in this particular case). These curves were obtained in the following way. The silver precipitation of Kossel was carried out quantitatively on the filtrate of a spleen completely autolysed at p_H 5. Both fractions were then evaporated to the original bulk.

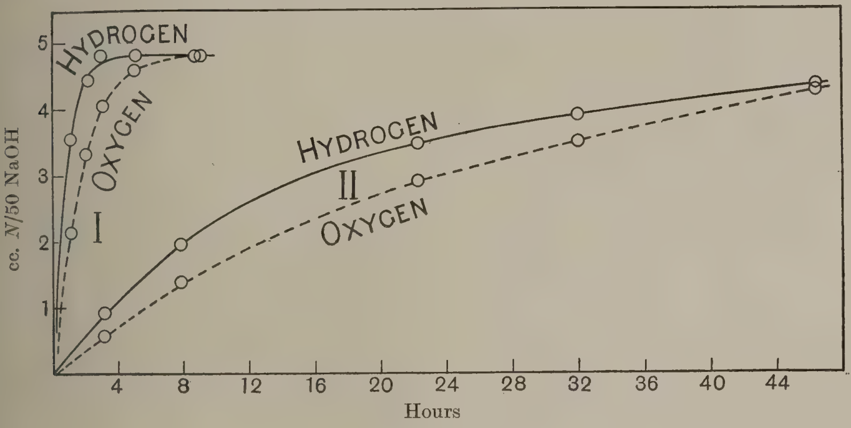


Fig. 4.

Curve I shows the urea production from the basic fraction; Curve II from the solution obtained by mixing equal volumes of both the basic and mono-amino fractions, and evaporating the whole to the volume of each before mixing. This is practically the composition of the original autolysate. The method of obtaining the curves has already been described in detail above. Both sets were of course taken at the same time with similar amounts of enzyme and under identical conditions. A theoretical meaning of these results may be that, granted that adsorption of the substrate on the enzyme precedes decomposition, many or all of the amino acids may also be adsorbed, and prevent access of the urea precursors to the active surface of the enzyme.

On applying these results to autolysis, it is evident, from the nature of the case, that this inhibition due to the products of autolysis must become greater and greater as autolysis proceeds, and so, other things being equal, the rate of production of the urea will become slower and slower.

(2) At the reactions used for studying autolysis, due mainly if not entirely to the p_H being an unfavourable one, the enzymes producing urea become more or less rapidly inactive. To investigate this, effects due to (1) must be eliminated. This was done when testing the activity of the enzymes by using a substrate containing such a high amino acid content that the comparatively

small increase of amino acids due to autolysis of the source of the enzyme during the experiment could be neglected. Such a substrate is spleen autolysed completely at p_H 5, boiled, filtered, and neutralised, which treatment gives almost as high a ratio $\frac{\text{Amino N}}{\text{Total N}}$ as is obtained by an acid hydrolysis. Liver, containing active urea-forming properties, was made up for autolysis at the p_H to be investigated, strained through muslin, and placed in the incubator at 37°, samples being withdrawn at once and again at suitable intervals, and the activity of the urea-producing enzymes tested. For this purpose 5 cc. substrate, 0·5 cc. of the liver suspension, and 10 drops of toluene were placed in a test-tube, and incubated 16 hours at p_H 7 and 37°, heated to 100° and the urea content estimated. Previous trial had shown that for the first 16 hours the rate of production of urea was approximately constant, reduction of rate due to lack of substrate not taking place till later. The activity of the enzymes producing urea falls off steadily from day to day, the rate depending on the p_H. Table I will make this more clear, the amount of urea formed in the 16 hours being taken as a measure of the activity of the enzymes, and their activity on the first day being reckoned as 100. Every test of activity was done in duplicate.

Table I.

p_H of autolysis, 6.

Days after setting up autolysis	Temp. of autolysis	Activity of the urea-producing enzymes
0	37°	100
2	,,	88
5	,,	76
8	,,	55

The steady fall in the activity of the enzymes shows that the effect is not due to accumulation of the products of autolysis in the liver suspension. Were it so, the rate of inactivation would be rapid at first and later fall off to a small figure.

The decline in the activity of the enzymes producing urea is much more rapid at p_H 5 than at 6, the activity being zero after three days. This is in keeping with the fact that the production of urea in liver autolysing at p_H 5 invariably ceases after three days. Even when freshly made up, the activity of the urea-producing enzymes of the liver made up at p_H 5 is never so great as that made up at p_H 6, though the p_H at which the activity is being studied is the same in both cases (p_H 7). The reason for this is probably the fact that phosphates accelerate the action of arginase [Gross, 1920], which certainly accounts for some of the urea formed under these conditions, and while phosphates were used to buffer the liver to p_H 6, phthalates were used for p_H 5.

It is now possible to define the conditions which must exist in order to obtain the inhibition by oxygen gas of the formation of the urea in autolysis, and to explain the apparent contradictions between the liver and the spleen results. The precursors of the urea must be set free by the enzymic hydrolysis of the tissue proteins at a rate faster than they can be decomposed by the urea-forming enzymes when inhibited by oxygen gas. In other words, the factor limiting the velocity of urea-formation must be the activity of the urea-producing enzymes themselves. In the spleen this is ideally obtained about p_H 6. The protein hydrolysis is rapid and fairly complete; the enzymes producing urea are never very active in this organ, and at p_H 6 their power to form urea becomes progressively hampered by the accumulation of the products of autolysis, and the loss of activity due to the p_H. The result is that the autolysis in oxygen shows a smaller total production of urea than its control in hydrogen, the enzymes producing urea in oxygen becoming inactive before they have time to decompose as much of their substrate as their fellows in hydrogen. The liver on the other hand possesses urea-forming systems many times more active than those of the spleen. At p_H 6 the precursors of the urea are never set free fast enough to allow the inhibition by oxygen to become apparent, for even when inhibited by oxygen the production of urea keeps pace with the liberation of the precursor. The limiting factor is the rate of tissue hydrolysis, and therefore the rate of urea production and the amount of urea produced are the same both in hydrogen and oxygen in this case.

In the spleen autolysing at p_H 5, the conditions are so unfavourable for the production of urea (small concentration of enzyme, low p_H, very rapid hydrolysis of the tissue proteins with accumulation of end products) that the action of the enzymes producing urea never becomes apparent. But in the liver autolysing at p_H 5, the conditions are just right for the inhibition by oxygen to show itself. The factor limiting the formation of urea is the activity of the urea-producing enzymes as in the spleen at p_H 6. Their higher concentration is balanced by a correspondingly greater inhibition due to p_H.

Effect of combining the actions of urease and the urea-forming enzymes.

Spleen autolysed to completion at p_H 5, boiled, filtered, and neutralised was used as substrate, 10 % suspension of liver, of the mucous lining of a dog's stomach or of soya bean meal being used as the source of the enzymes.

Liver suspension plus urease acting together were always found to produce 25 % more urea (as NH_3) than liver acting alone. The tubes were incubated three days at 37° and after heating to 100° urease was added to each for 10 mins. at 40° before estimating. However, on arranging the experiment differently it was found that liver plus urease acting together for three days gave exactly the same amount of urea (as NH_3) as urease (alone) for three days *followed* by liver (alone) for three days. (After the first three days the urease was destroyed by boiling.)

This probably means that the substrate contains small amounts of the urea precursor still in a combined form which the proteolytic enzymes of crude soya bean meal and the mucous lining of a dog's stomach can liberate, while the liver, though unable to liberate the precursor, alone possesses the means of decomposing it.

SUMMARY.

(1) That oxygen gas markedly inhibits urea production in autolysis has been confirmed.

(2) The effect of oxygen is merely to inhibit the rate of urea production by tissue enzymes, but in autolysis where other factors (p_H, accumulation of amino acids, etc.) are in operation simultaneously, the effect of oxygen may also be to reduce the total amount of urea formed.

The author wishes to express his gratitude to Professor Hopkins for his advice and criticism.

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XX. THE INFLUENCE OF STORAGE ON THE ANTISCURVY VALUE OF FRUITS AND VEGETABLE JUICES.

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(*Received December 23rd, 1924.*)

In recent years, attempts to place our knowledge of food values on a wider scientific basis have led to a series of researches on the effects of cold storage on a variety of products. One of the earliest of these was an investigation started in 1918 at the Lister Institute which was organised by Dr H. Chick and assisted by a grant from the Food Investigation Board. The object of the experiments was to find out the effects of prolonged storage under various conditions on certain common fruit and vegetable juices known to be of considerable antiscorbutic value when fresh. The tests have been carried out from time to time by a number of workers, all following the same methods and with a considerable amount of co-operation. Preliminary results with chilled fruits and fruit juices stored at various temperatures were published by Davey from the Lister Institute in 1921. The continuation of the work was interrupted however owing to unforeseen circumstances and the results have not yet been presented in a collected form. The research begun at the Lister Institute by Davey and others was continued by me in 1923 at the Biochemical Department, Institute of Physiology, University College and in the following year at Westfield College, in the Department of Botany, with the help of a grant from the Food Investigation Board. I have to thank Prof. Drummond for his kindness in connection with the experiments at University College and Dr H. Chick for giving me free access to previous records at the Lister Institute and for kindly reading the proofs of this article.

The technique of testing the juices is that described in detail by Delf and Tozer [1918], following in the main that of Chick, Hume and Skelton [1918]. Young guinea-pigs weighing at first about 320 g. were fed on a basal diet of crushed oats and bran together with a ration of 60 to 90 cc. of cow's milk previously autoclaved at about 120° for one hour. The fruit juices to be tested were fed by hand as daily doses and the smallest dose which effected protection from scurvy over an experimental period of 90 days was taken as the minimal protective ration for comparative purposes.

I. Cold storage of fruits and vegetables or their juices.

(a) *The storage of chilled fruits and vegetables.*

Oranges, lemons, tomatoes and swede-turnips were selected for trial as being easy to obtain in quantity and also rich in their content of vitamin C. Only carefully selected sound material was used. Each specimen was wrapped in paper to prevent contact with its neighbour, packed in a crate and conveyed at once to a chilling room of the Union Cold Store, Red Bull Wharf, E.C., whose proprietors kindly placed a chamber in their establishment at the disposal of the Lister Institute for this research.

(1) *Oranges.* A number of selected Denia oranges were packed and placed in the chilling room (temp. 2·5–5·4°) on May 7, 1919. They were inspected in July and again in October the same year when feeding experiments were started. At this time 96 oranges were still available of which only 11 were sound. The worst fruit was used first, the more rotten parts being cut away and the juice of the remainder squeezed as required through muslin. It was shown by Davey [1921] that the minimum protective dose of this juice was equal to that of the fresh raw fruit, being only 1·5 cc. daily. The storage and partial decay of the fruit had thus not appreciably impaired the antiscorbutic value of the juice.

(2) *Lemons.* Lemons packed and placed in the chilling room about the middle of April 1919 were inspected in July and November of the same year and were finally removed and tested in January 1920. Their condition was worse than that of the oranges, none being sound at the beginning of the experiment. The juice of these was fed to the experimental animals without previous neutralisation and frequently unsound parts of the lemons were used so that the juice was often discoloured. The daily doses were 1·5 cc., 2·5 cc. and 5 cc. respectively. In every case severe scurvy resulted, 51 days being the longest time of survival; little if any protection was therefore received.

The conclusion drawn from these experiments was that the juice of oranges and probably also of lemons does not lose appreciably in antiscorbutic value as long as the fruit is fit for food, but that the fruit cannot be kept in the chilled condition for more than four or five months.

(3) *Swede-turnips.* On May 10, 1919, swede-turnips were procured, some from Covent Garden and some from Newcastle through the kindness of Prof. Hutchens. Each consignment was examined immediately on arrival, only sound roots being used for the chilling room. The swedes were inspected in July and again in October of the same year. On the latter date, only a dozen of the Covent Garden batch remained sound; those from Newcastle were all more or less rotten: a few however were brought to the Lister Institute and tests were begun on the following day. They were only continued for 50 days owing to the rapid decay of such swedes as remained and towards the end of the experiment juice from unsound parts had to be included in the doses fed to the animals. Two animals on a dose of 2·5 cc. developed severe scurvy, this amount being

a sufficient ration in the case of the fresh juice. Two animals, each receiving 5 cc., were chloroformed after 35 days owing to lack of further juice. One showed typical scorbutic lesions while the other was normal. This experiment is inconclusive with regard to the effects of "chilling" since on the second visit to the store, some of the roots were found frozen which probably accounted for the rapid decay of the tissues. It is also inconclusive with regard to the antiscorbutic value of the juice, since protection was not attained over any appreciable experimental period. However, the results are not inconsistent with the conclusion derived from the consideration of the chilled oranges and lemons.

(4) *Tomatoes.* On August 6, 1919, a similar attempt was made to preserve tomatoes in the chilling room of the Union Cold Store. These were in fine condition when taken; they were examined in October the same year, and seemed mostly sound; a few with dark spots were carefully isolated with folds of paper. Examined again in January 1920, all the fruit was found to be spoiled and no further attempt was made to repeat the experiment.

(*b*) *Storage of fruit and vegetable juices.*

For commercial purposes, fruit juices are often preserved by the addition of a small proportion of some substance such as potassium metabisulphite. At low temperatures, however, the juice may be kept without the addition of any preservative and early in 1919, experiments were started to determine the influence of prolonged cold storage on the juice of oranges, lemons and swedes respectively.

(1) *Lemon.* On March 27, 1919, a number of sound lemons were well washed, dried and squeezed, the juice being then passed through muslin and placed in small glazed earthenware jars of about 150 cc. capacity, which were then at once corked and sealed with wax. A number of these jars were immediately stored in a cold chamber of the Colonial Consignment and Distributive Co., Ltd., which was generously set aside by them for the purpose. The temperature was checked by means of an automatic recording instrument and only varied from -11 to $-14°$. When samples of the juice were withdrawn for testing, they were always found to be frozen.

This juice remained undisturbed until August 1920 when test experiments were started at the Lister Institute by Davey, a jar of the frozen juice being fetched every fortnight for the purpose. Two animals each receiving a daily ration of 1·5 cc. of this juice remained free from scurvy for 90 days. This is no more than the minimal daily amount of the fresh juice giving protection from scurvy; there was thus no appreciable loss of vitamin during the first 17 months of cold storage.

In February 1924 some of the remaining juice was tested, jars of juice being fetched once a week. Three animals each receiving 1·5 cc. of the juice early began to show typical symptoms of scurvy. When these became severe, the ration was increased to 2·5 cc. One showed no improvement and was chloroformed after 38 days; the other two were given a further allowance (2·5 cc. for 12 days, 3 cc. for the last 51 days). At the post-mortem, all these showed typical scorbutic lesions, severe in the first case, and nearly healed in the case of the two on increased doses. Three other animals on a daily ration of 3 cc. throughout the experiment remained in perfect health and showed no sign of scurvy at post-mortem examination.

These experiments show clearly that during five years' storage in the frozen state, a slow deterioration takes place, a minimum ration of nearly 3 cc. being required for protection instead of 1·5 cc. the amount needed of the raw fresh juice. Of the 135 jars originally stored only 22 have been used, the rest being still buried under an accumulation of snow in the freezing chamber. By prolonging the curve B (Fig. 1) smoothly, it will be seen that if the deterioration continues in the same way, a dose of about 5 cc. given in 1926 (*i.e.* after seven years' storage) should just suffice for protection.

(2) *Orange.* On March 13, 1919, orange juice was prepared, sealed and taken to the same cold storage chamber as the lemon juice. The first tests were made by Davey beginning in August 1920. As in the case of the lemon juice, a daily dose of 1·5 cc. was found to give complete protection and no measurable deterioration had occurred after 17 months of cold storage.

In February 1924 further tests were carried out by the writer, after five years of storage. Three animals on the usual basal diet were each given daily doses of 1·5 cc. of the juice. After 40, 42 and 49 days respectively, symptoms of scurvy became defined; the doses were then increased until at the end of the whole period the average ration per day amounted to 2·5 cc. At post-mortem examination all were found to have enlarged rib-junctions and dark coloured muscles; and one had also small lesions in the tissues around the knees and rib-junctions.

Three other animals kept on a uniform ration of 3 cc. survived for 91 days in good condition, but with occasional symptoms of scurvy. At the post-mortem examination, two had somewhat brittle bones, with some of the rib-junctions beaded; the third had in addition small petechial haemorrhages over the rib-junctions of both right and left side. This dose may therefore be taken as marginal in value. From these results, it appears that this sample of orange juice is perhaps somewhat less stable than the lemon (Fig. 1, A, B).

(3) *Swede-turnip.* The swedes for this experiment were obtained, some from Covent Garden and some from Newcastle as for the storage of the whole roots by chilling. The swedes were washed, grated on an ordinary kitchen grater, and the resulting pulp squeezed through muslin. The juice was placed in small glazed earthen jars of about 70 cc. capacity and was stored with the orange and lemon juice on 14th May 1919.

In August 1920, after 15 months' storage, the frozen juice was tested at the Lister Institute by Davey. Two animals each receiving 2·5 cc. of the juice developed acute scurvy and were chloroformed in an advanced condition after 37 days. Two others each receiving 5 cc. developed symptoms of scurvy on the 26th and 28th days respectively. Both were chloroformed after 35 days of experiment and the post-mortem examination showed typical signs of severe scurvy. Two other animals were given 10 cc. daily beginning in October 1920; both became very ill with scurvy complicated by intestinal trouble, and had obviously received no protection. The administration of larger doses of the juice was not practicable.

Swede juice thus differs materially from the juice of oranges and lemons in that after only 15 months of cold storage in the frozen condition practically no antiscorbutic value remains. Some of this juice remains still in the freezing chamber and might be worth comparison with the fresh juice from the chemical point of view.

Table I. *The relative antiscurvy value of fresh and stored frozen juices.*

	Minimum protective ration cc.	Loss of vitamin C
Orange, fresh, raw	1·5	
Stored, frozen:		
1 year 5 months	1·5	Not appreciable
5 years	$>$2·5	More than two-fifths
	about 3	About half
Lemon, fresh, raw	1·5	
Stored, frozen:		
1 year 5 months	1·5	Not appreciable
5 years	$>$2·5	More than two-fifths
	$<$3	Less than half
Swede, fresh, raw	2·5	
Stored, frozen:		
1 year 3 months	$>$ 5	Much more than half
1 year 5 months	$>$10	,, ,, three-quarters

In the series of experiments described above, uniformity of material was secured as far as possible by the choice of ripe sound fruit, and by testing samples of the same material before and after storage. This is of some importance, since it is known that the antiscurvy value of the fresh product is itself subject to a good deal of variability. It has been proved for instance that the antiscorbutic value of orange juice diminishes somewhat towards the end of the season [Davey, 1921, p. 89], and that young carrots are more effective than old ones [Hess and Unger, 1919]. How far these and other variations in the condition of the fruit also affect the keeping properties is almost entirely unknown. Recent researches on the cold storage of apples show that in at least one variety, the time of survival in cold storage varies greatly, according to the age of the tree, the orchard and the season in which the crop was grown [Haynes, 1924].

The striking contrast between the behaviour of orange or lemon and swede juice calls for further comment. An obvious difference lies in the acidity. Acid fruit juices are found to be considerably more stable than neutral ones as regards antiscorbutic properties. Recently Zilva [1923, p. 416] has found that when decitrated concentrated lemon juice has part of its acid restored there is much less loss of antiscorbutic properties on storage. According to La Mer and Campbell [1921] and La Mer [1921] the degree of acidity in tomato juice also affects its stability when exposed to rise of temperature. However, different juices of about the same natural acidity may have very different degrees of stability in their antiscorbutic properties, *e.g.* swede juice is more stable to heat than cabbage juice, and orange juice nearly neutralised as tested by Delf [1920] is much more stable than tomato juice neutralised to about the same extent as described by La Mer [1921].

In the preparation of the orange, lemon and swede juice for storage, no attempt was made to exclude air and slow oxidation doubtless occurred. Onslow has recently shown that in many fruits (apple, plum, pear) the cell sap contains enzymes and their substrates forming an autoxidising system which is incomplete in the case of orange, lemon and various other acid fruits. It is possible therefore that the greater destruction in the swede juice may be due to some kind of autoxidation in the juice, but there is no direct evidence as yet upon this point.

II. Storage of canned fruits (oranges and tomatoes).

(*a*) *Oranges*. In April 1919, a number of Denia oranges were peeled, divided into convenient parts and packed into cans[1]. These were processed for 20–30 minutes, the temperature rising gradually to 100° and remaining at 100° for not more than five minutes. The cans were then sealed, cooled and stored at laboratory temperature. Five months later, the first test was made. Allowing for the small amount of water added to the fruit after packing the cans, a dose equivalent to 1·5 cc. of the fresh juice was found to give adequate protection from scurvy. No loss of antiscorbutic activity was therefore indicated at this time.

A further test was made after four years' storage, beginning on June 6, 1923. Three animals each receiving daily 3 cc. of the juice escaped from scurvy, but at post-mortem examination the muscles were of a very dark colour, and in two cases small periosteal haemorrhages were seen in the regions of the costochondral junctions. These doses were measured without allowance for the water added in canning and represent 1·5–1·7 cc. of the original juice. Animals on a higher ration (equivalent to 2·5 cc.) were completely protected from scurvy. The canned orange juice has thus lost but a small proportion of its original antiscurvy value, after storage for more than four years. If the minimum protective ration be plotted against the time of storage, it appears that the canned juice stored at laboratory temperature is more stable than the raw juice kept at − 10° to − 14°. In view of this result, it seems probable that swede juice could also be successfully stored if previously heated and sealed in absence of air. This has only a theoretical interest, but it would probably apply equally to fruits such as pears and plums which are frequently bottled but the antiscorbutic value of which is little known.

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[1] The first batch was prepared by Miss Campbell; the rest by myself; the testing was done by myself.

(*b*) *Tomatoes.* The effect of storage on canned English tomatoes has been recently described [Delf, 1924]. The minimum protective dose of the raw juice lies near 1·5 cc. (estimated at about 2 cc.); that of the freshly canned tomato was about 8·5 cc.[1], this deterioration being in marked contrast to that which occurred with orange juice. After storage for four years, a dose averaging 10 cc. daily gave almost perfect protection for 68 days. For the full experimental period of 90 days a slightly increased ration would probably have been needed. The deterioration caused by storage is slow but is greater than that in the case of heated orange juice (Fig. 1). The reason for this is not clear since both are acid fruits and were canned and stored in the same way. As a matter of fact, it was noticed that the tomato juice when removed from the tins on opening did not keep as well as the orange juice especially in the hot weather. For the most part, juice showing signs of fermentation was rejected, but owing to the small supply fresh tins of canned tomatoes could not be opened as frequently as desired especially towards the end of the experiment.

Table II. *The relative antiscurvy value of freshly canned and stored canned fruit.*

	Minimum protective ration	Loss
Orange juice:		
Fresh, raw	1·5 cc.	
Canned, newly	1·5 cc.	Not perceptible
Canned and stored 4 years at lab. temp. ...	1·7 cc.	About 10 %
Tomato:		
Fresh, raw	1·5–2·5 cc. Estimated at about 2 cc.	
Canned, newly	7·5–10 cc. Estimated at about 8·5 cc.	About 75 %
Canned and stored 4 years at lab. temp. ...	>10 cc.	Over 80 %

III. Storage of raw lemon juice with added preservative.

In 1918 a quantity of lemon juice was prepared for storage by the addition of potassium metabisulphite as described by Davey [1921].

(*a*) *Material preserved with ·06 % sulphite.*

Two lots of this material were made up. The first made in February 1918 was tested after four and again after six months, but the results were not satisfactory owing apparently to exposure to air during the intervals of the experiment, and are set aside for the purpose of the present discussion. A further test on the second lot of material was made at intervals varying from 18 to 22 months. The experiments were not quite finished at the time when the account by Davey went to press, but apparent protection was given by doses of 2·5 and 5 cc., no post-mortem examination being made in either case. In 1923 a further test was made by me at University College. No special precautions were taken to exclude air from the stock bottle which was kept at laboratory temperature throughout the experiment. No deterioration seemed to occur either in the appearance or taste during this time.

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[1] According to Hess [1921] and La Mer [1921] bottled tomato juice in America has the much lower minimum protective ration of 3 cc.; the juice used by La Mer was prepared by a well-known canning company and was processed for a much shorter time than that prepared and used at the Lister Institute.

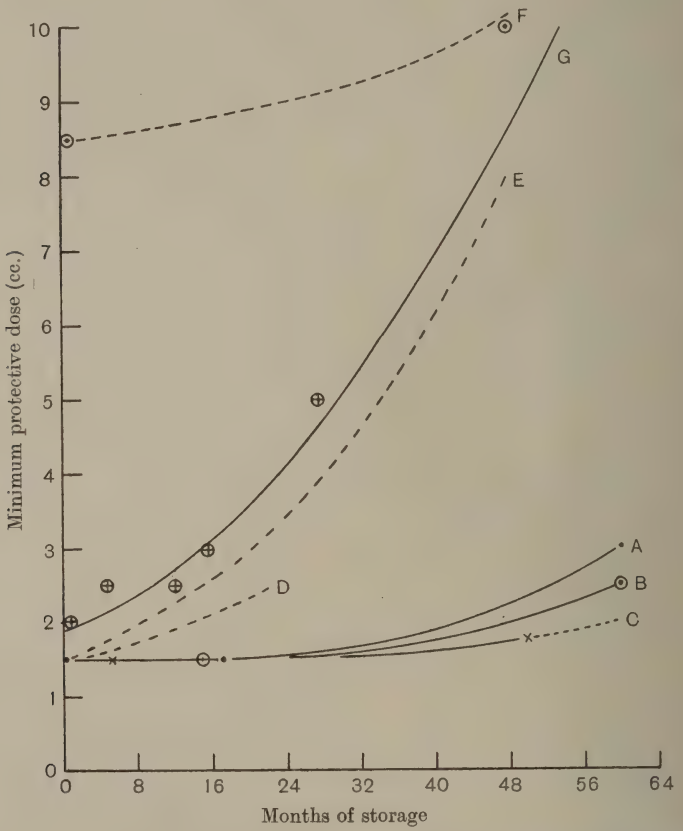

Fig. 1. Diagram showing relation between time of storage and minimum protective ration.

(A) Frozen orange juice (·—·), data as in Table I.
(B) Frozen lemon juice (⊙——⊙), data as in Table I.
(C) Canned orange juice (×—×), data as in Table II.
(D) Sulphite lemon juice ·06 % (cold room), data as in Fig. 2, D.
(E) Sulphite lemon juice ·06 % (lab. temp.), data as in Fig. 2, E.
(F) Canned tomato juice (⊙------⊙), data as in Table II.
(G) Lemon juice with rind oil (lab. temp.) (⊕—⊕), data as in Table III.

Note. The curves D and E are taken from those in Fig. 2 of the same lettering, where the amount of approximation is shown on the same scale.

Three strong young guinea-pigs were selected for the purpose of the test. They disliked the juice so much that for the first 12 days the ration was kept at 5 cc., after which it was increased to 10 cc.[1] fed in two daily instalments with other food between. The animals made fairly good growth and escaped typical scurvy but often showed signs of tenderness in the hind limbs. At post-mortem examination, after 88 days of experimental diet, one of the three was found to be quite normal; one had a haemorrhage into the wall and cavity of the middle region of the small intestine and the third had two small periosteal haemorrhages near the costochondral junctions. In every case the lower molars and in one case the tibiae also were markedly brittle. This dose, averaging 9 cc. daily over the whole period, therefore gave apparent protection, which, as seen from the post-mortem, was very nearly marginal. The undoubted value of the juice in this case suggests that this sulphite sample stored at laboratory temperature had a considerable degree of stability. It is obvious, however, that it is inferior in this respect either to frozen juice (Fig. 1, cp. E, B) or to the same juice stored at a lower temperature (Fig. 1, D).

A sample of this preparation was also stored at about + 4° and was examined after 27 months [Davey, 1921].

The results of these and other experiments are collected in Table III. Assuming the process of ageing to be an orderly one and making allowances for the approximate nature of some of the results, they may be expressed graphically (Fig. 2). The general form of the curve E is defined by the determination of the minimum dose after $4\frac{1}{2}$ years' storage; the other points are less well known, but unless there was any initial rapid loss of antiscorbutic value on adding the preservative (a point which was not investigated at the time) the course of deterioration might be expected to follow some such direction as the line suggested in the smoothed curve E. The curve F is not so well defined since the final test failed to give protection; from what has been said about the relative value of the other doses, it cannot be very different from that indicated and the continuation of it must at least indicate a rate of deterioration considerably greater than that of the juice with a smaller amount of added preservative (curve E). This is clearly in opposition to the tentative conclusion of Davey [1921, p. 98] that the larger amount of preservative was probably to be recommended.

(*b*) *Material preserved with* 0·09 % *sulphite.*

Results of the earlier experiments with juice having a final concentration of 0·09 % added sulphite were briefly indicated by Davey [1921], but the experiments were unfinished at the time of publication. After seven months' storage, a daily dose of 5 cc. gave protection but no lower dose was then tried. After 12 months, three animals on a dose of 2·5 cc. received partial protection, symptoms of scurvy becoming well defined but not developing until the end of the experiment. The three animals on this dose behaved differently, one dying with severe scurvy after 61 days, another showing no symptoms until after 66 days and the third having no symptoms until after 94 days. In the case of the last animal the scurvy was of a definite but mild type although the experiment was prolonged for 100 days.

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[1] The average dose for the whole period was 9 cc., but since the lower ration was fed only at the beginning of the experiment, the result may be taken as due to a ration throughout of about 10 cc.

After 19 months' storage, a daily dose of 5 cc. gave considerable protection to each of two animals. The first developed symptoms after 78 days and died with severe scurvy after 84 days of experiment; and the second developed symptoms after 84 days and survived for 90 days, showing signs of mild scurvy at post-mortem. A dose of 10 cc. afforded complete protection in an experiment begun when the juice was 22 months old and continued for 86 days.

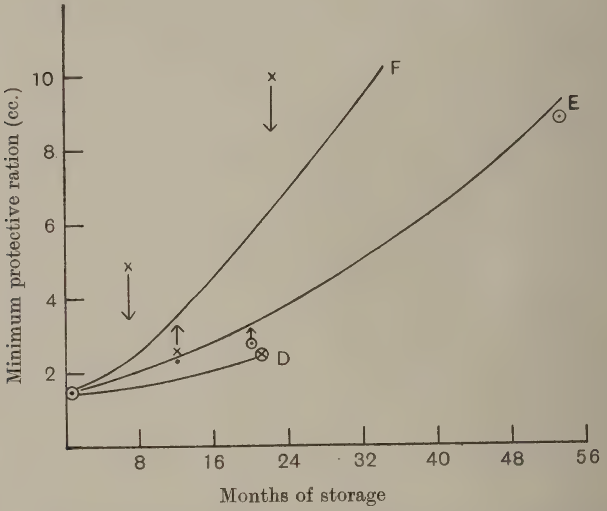

Fig. 2. Diagram showing the relation between time of storage and probable minimum protective ration; lemon juice with added sulphite:

·06 % stored at laboratory temperature, (⊙ ⊙) E.
·06 % stored in cold room (4–10°), (⊗ ⊗) D.
·09 % stored at laboratory temperature, (× × ×) F.

In June 1923 an attempt was made by me to apply a further test, after $4\frac{1}{2}$ years of storage at laboratory temperature. Three animals were started on a daily dose of 5 cc., but owing to difficulties of feeding the ration was not further increased. Symptoms of scurvy were noticed on the 52nd, 53rd and 67th day respectively. The symptoms were progressive, the last animal surviving for only 80 days, when it was chloroformed in an advanced stage of severe scurvy. No larger doses were practicable, but a consideration of all the results seems to show that the higher percentage of sulphite is not conducive to the preservation of the antiscorbutic value of the juice (cp. Fig. 2).

IV. The use of rind oil as a preservative.

The fresh juice of lemons and oranges as prepared with the rind oil by Davey in 1918 was stored part at laboratory temperatures[1] and part in the cold room of the Lister Institute. The results are given by Davey [1921] and are summarised for reference in Table III. In the summer of 1923, a further test was made using a daily ration of 5 cc. This gave partial protection to each of three animals which developed "chronic" scurvy, becoming severe but not fatal. It was impossible to feed larger doses at the time but some indication of the course of deterioration may be seen by plotting all the values found (Fig. 1, G).

Table III. *Lemon juice with added preservative.*

Condition	Temp.	Age	Minimum protective dose	
Raw	Laboratory	Fresh	1·5 cc.	
With ·06 % sulphite	,,	18–22 months	? >2·5–<5 cc.	Fig. 2, E
	,,	4½ years	9–10 cc.	
	Cold room	27 months	<5 cc. >2·5 cc.	Fig. 2, D
With ·09 % sulphite	Laboratory	7 months	< 5 cc.	Fig. 2, F
		12	> 2·5 cc.	
		19	> 5 ,,	
		22	<10 ,,	
		4½ years	> 5 ,,	
With rind oil	Laboratory	Fresh	>1·5<2·5 cc.	Fig. 1, G
		4½ months	<2·5 cc.	
		12	<2·5 ,,	
		27	5 ,,	
		5 years	>5 ,,	

A similar set of experiments over a shorter period was carried out using orange juice. The stored orange juice behaved in much the same way as the lemon juice [Davey, 1921] but there is not sufficient evidence to make a detailed comparison of the stability of the two juices.

General conclusions.

(1) When whole fruits (oranges or lemons) or tuberous roots (swedes) are stored, their antiscorbutic value is retained as long as the tissue itself remains living.

(2) The expressed juice of oranges and lemons stored for five years in the frozen condition retains about half its original antiscorbutic potency. Swede juice on the other hand stored in a similar way, loses the greater part of its value in less than two years. It is suggested that the greater deterioration in the latter case may be due to the lower acidity or to autoxidation or to both.

(3) Canned orange and tomato juice retain much of their value even after storage for four years at laboratory temperature. The tomato as prepared for these experiments was much less stable than the orange.

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[1] For the first 10 months all the juice was kept in the cold room; later part was removed and stored at laboratory temperature.

(4) Lemon juice with added potassium bisulphite retains its flavour well after storage for $4\frac{1}{2}$ years at laboratory temperature. At this age, the protective dose of lemon juice containing ·06 % sulphite was about 10 cc. instead of the original 1·5 cc., thus indicating a loss of about five-sixths of the original value.

(5) Both orange and lemon juice can be preserved satisfactorily for one to three years at laboratory temperature in presence of their own rind oil.

(6) Inspection of the comparative curves in Fig. 1 shows the relative stability of the juices examined. Canned orange is the most stable and this is in agreement with previous findings with heated juices [Delf, 1920]. The frozen juices are next in order of stability; the form of the curves in preserved juices suggests that the added sulphite has a destructive effect on the stability of the antiscorbutic vitamin.

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XXI. A CHEMICAL STUDY OF DEFECTIVE OSSIFICATION IN RACHITIC ANIMALS.

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Any attempt to understand the mechanism of ossification leads naturally to a consideration of those conditions which give rise to abnormalities in bone development. Apart from their intrinsic interest the investigation of such cases may throw light on the process as it takes place in the normal individual and it was for such reasons that the present study of ossification in rachitic animals was commenced.

Among the very large number of published papers on the cause and nature of rickets, relatively few deal with the chemical aspects of the defective ossification, the immediate cause of the failure to deposit the normal amount of calcium salts in the rachitic bone. Some fairly complete analyses of the inorganic constituents of normal and rachitic bones have been published, and the concentration of calcium and phosphate in the blood has been studied by a number of investigators. While their evidence shows that in children and in animals, rickets is frequently associated with a lowered content of inorganic phosphate in the blood, this is not always the case and is not specific of the condition [Hess and Unger, 1922]. There is little evidence of any significant variation in the concentration of total calcium [Howland and Kramer, 1921]. It would seem that the investigation must go deeper if this complex chemical problem is to be solved.

On the basis of the suggestions put forward in previous papers [Robison, 1923; Robison and Soames, 1924[1]] the deposition of calcium phosphate in the growing bone would be adversely affected by any of the following factors:

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[1] *Correction.* In our previous paper [Robison and Soames, 1924] the equation (1) on p. 752 (and the same equation on p. 749) should read

$$[Ca^{++}]^3 \times [PO_4^{\equiv}]^2 = K_1 [Ca_3(PO_4)_2].$$

The limiting value of $[H^+]$ below which $Ca_3(PO_4)_2$ would be deposited (p. 752) is $K_3\sqrt{\frac{k_2^3}{k_1[HPO_4^{=}]}}$.

The conclusion that this value depends on the respective solubility products and the dissociation constant of the acid phosphate ions is not affected although for this particular case it would also depend on the relative amounts of calcium and phosphate in solution. The case is, however, a hypothetical one for, as previously stated, the solid phase is neither $CaHPO_4$ nor $Ca_3(PO_4)_2$ but a complex basic phosphate of whose ionisation we have no certain knowledge.

1. Lowering of the concentration of calcium ions in the plasma either as the result of a diminution in the total calcium content or of a disturbance in the equilibrium between the ionised and un-ionised portions thereof.

2. Lowering of the concentration of the phosphate ion corresponding with the bone phosphate (probably hydroxyapatite), either as a result of a diminution in the total inorganic phosphate or of a shift in the equilibrium between the various phosphate ions (change of p_H).

3. Failure of the cartilage and bone cells to secrete the enzyme.

4. Deficiency in the supply of the phosphoric ester which is normally present in the blood and is hydrolysed by the bone enzyme.

5. Alteration in the permeability of the corpuscular membrane, capillary wall or cartilage.

6. Failure of the mechanism (if such exists) by which the p_H of the tissue fluid is raised within the cartilage.

The occurrence of either (1) or (2) unaccompanied by an equivalent rise in the concentration of the other ion would result in the product of the ionic concentrations falling below the solubility product (k) of the bone phosphate and the plasma becoming unsaturated with respect to this substance. Deposition of the phosphate might still occur if the local increase in the inorganic phosphate resulting from the enzymic hydrolysis of the phosphoric ester were sufficient to raise the product of the ionic concentrations above k, but the amount of the calcium salt deposited would be reduced.

Failure either of the enzyme or of the specific phosphoric ester would result equally in deficient calcification since the necessary local increase in the phosphate concentration would not occur.

The phosphoric ester is present chiefly in the corpuscles and the conditions under which it passes into the plasma and cartilage fluids are not fully understood, but it is clear that the supply of this ester to the site of ossification might fail owing to changes in the permeability of the membranes (of the corpuscles etc.) even though the total amount present in the whole blood was not reduced.

It is conceivable also that changes in permeability of the cartilage or of the capillary walls might, by affecting the Donnan equilibrium, alter the relative concentrations of calcium and phosphate ions in the plasma and cartilage fluid.

The participation of the bone enzyme in the ossification process has been supported by a considerable weight of evidence but the further suggestion of a possible mechanism by which the p_H is locally raised is a teleological speculation and arises mainly from the evidence of the high optimum p_H of the enzyme. No direct proof of such a mechanism has yet been found. Its effect would be to increase the activity of the enzyme and, independently of this, to favour the deposition of calcium phosphate and of calcium carbonate.

It is obvious that a deficiency either of calcium or of phosphate in the diet, resulting in a lowered concentration of these substances in the blood, will adversely affect calcification of the skeleton and may be a contributory cause of this defect in rickets, but the evidence at present appears to show that in well marked cases of rickets the concentration both of total calcium and of inorganic phosphate in the blood may be normal. A considerable proportion of the calcium is certainly present in un-ionised forms and will play only an indirect part in determining the precipitation of calcium salts. The investigation of the relation between the ionised and un-ionised calcium in plasma is a difficult but very attractive problem which we have not touched in the present paper but are hoping to attack later.

The inorganic phosphate is probably very largely ionised but the concentration of the particular anion corresponding with the solid phase (bone phosphate) must in any case be very small. It will be affected chiefly by the p_H of the fluid but to some extent also by the various cations present.

The bones of rachitic rats have been shown to contain at least as much enzyme as normal bones [Robison, 1923] and have been used to demonstrate the deposition of calcium phosphate *in vitro*, by immersing them in solutions of phosphoric esters. Failure to calcify is certainly not due to lack of enzyme. A deficiency in the amount of the specific phosphoric ester was however an attractive possibility and its investigation formed the chief part of the work here described.

EXPERIMENTAL.

Effect of Diet.

The first series of experiments was planned and carried out early in 1923 in collaboration with Dr Goldblatt before it was known that the organic acid-soluble phosphorus compounds in blood consist of at least two esters, only one of which, the smaller fraction, is hydrolysed by the bone enzyme [Kay and Robison, 1924].

A considerable number of rats was divided into groups and fed on various diets deficient in the fat-soluble factor, calcium or phosphorus or in two of these constituents, normal controls being provided for each group. When growth had ceased or rickets developed the animals were bled and killed under ether anaesthesia and estimations of the inorganic phosphate and total acid soluble phosphorus in the whole blood were carried out by the Briggs modification of the Bell-Doisy method. The fact that the inorganic phosphate is increased *in vivo* during ether anaesthesia and *in vitro* when the blood is laked with water was not discovered until after these experiments were completed [Martland and Robison, 1924] and it is very probable that the values obtained were all about 10 % too high. The averages for each group should however be comparable. The values for the total P should be correct. The results are shown in Table I. Except in the case of those rats which were fed on the McCollum diet there is no evidence that the deficiency of the fat-soluble factor results in a lowered concentration of inorganic phosphate in the whole blood or that the addition of cod-liver oil to such a diet causes any appreciable increase. The average organic acid-soluble phosphorus is, however, regularly increased by the addition of cod-liver oil to the diet, although the amount of the increase is small and within the limits of variation found in individual rats on the same diet.

The rats fed on the McCollum diet No. 3143 showed an abnormal behaviour in this respect. The inorganic phosphate level was markedly below that found with any other of these deficient diets but was raised by nearly 70 % when cod-liver oil was given, although the total P did not change. The values obtained are not unlike those found by other investigators using this diet.

The low availability of the phosphorus derived from the cereals in this diet is well shown by comparison of the results obtained with those of Group IV in which the animals received a much smaller amount of phosphorus but chiefly in the form of inorganic phosphate, derived from the marmite. The concentration of inorganic phosphate in the blood was here nearly normal and was not increased by the addition of cod-liver oil to the diet.

Diets.

F.	Heated casein, starch, cotton seed oil, marmite, lemon juice, salt mixture, mixed to a stiff paste with distilled water.
G.	Same as F, with omission of calcium from the salt mixture.
E.W.	Cooked egg-white replaced casein in diet F and a salt mixture free from phosphate was used.
McCollum 3143.	Wheat, maize, wheat gluten, gelatin, calcium carbonate, sodium chloride, mixed to a stiff cake with distilled water.

Table I.

			% of Ca and P in diet as given to animals		Acid-soluble P compounds in 100 g. whole blood		
Group	No. of rats in group	Diet	Ca	P	Inorganic mg. P	Organic mg. P	Histology
I a	11	F (−A)	0·25	0·54	8·2	26·7	Mild rickets or osteoporosis
I b	11	F+C.L.O.	0·25	0·54	8·2	29·3	Normal with slight osteoporosis in some cases
II a	5	G (−A−Ca)	0·04	0·59	8·4	26·0	One normal, others with osteoporosis or osteomalacia
II b	3	G, +C.L.O. (−Ca)	0·04	0·59	8·5	28·4	Normal or osteoporosis
III a	5	E.W. (−A, −P)	1·08	0·08	4·5	20·5	Mild rickets or osteoporosis
III b	3	E.W.+C.L.O. (−P)	1·08	0·08	4·3	24·7	Nearly normal
III c	3	E.W.+C.L.O. +phosphate	1·08	0·54	5·8	27·3	Nearly normal
IV a	4	McCollum 3143	0·76	0·19	3·1	19·0	Rickets mild and florid
IV b	3	McCollum 3143 +C.L.O.	0·76	0·19	5·2	17·0	Nearly normal, some osteoporosis
IV c	3	McCollum 3143 +phosphate +C.L.O.	0·76	0·42	7·5	29·7	Nearly normal

Soon after these experiments were completed it was found that only a fraction (about 20 %) of the phosphoric esters is hydrolysable by the bone enzyme and it became apparent that increases (2–3 mg. P) such as were observed when cod-liver oil was added to the diets might, if occurring entirely in this fraction, represent very significant changes. A further series of experiments was therefore planned in which the amount of phosphoric ester hydrolysable by the bone enzyme should be estimated according to the method

Table II.

			Acid-soluble P compounds in 100 g. blood			Analysis of bones			
No. of exp.	No. of rats	Diet	Inorganic mg. P	Organic mg. P	Hydrolysed by bone enzyme mg. P	H_2O %	Ca % of dry bone	P % of dry bone	Ca : P
1	6	McCollum 3143	2·5	20·1	3·6				
	5	Do + C.L.O.	4·1	20·2	4·2				
9	3	McCollum 3143	2·6	24·9	5·1	62·5	12·11	5·40	2·24
	3	Do + C.L.O.	4·6	21·1	3·1	56·6	16·17	7·44	2·17
16	2	McCollum 3143	3·2	19·3	3·2	—	14·5		
	2	Do + 10 cc. milk	5·1	18·8	3·2	—	16·2		
18	2	McCollum 3143	2·1	24·3	4·5	54·8	16·30	7·22	2·26
	2	Do + oxidised C.L.O.	5·4	27·7	3·9	47·8	18·37	8·46	2·17
3	3	F	5·1	27·0	3·4	47·2	16·46	7·72	
	2	F + irradiation	5·1	27·6	4·4	44·6	20·36	9·27	
	2	F + C.L.O.	5·6	27·3	4·6	40·8	21·95	10·64	
5	4	F	5·1	26·6	5·6	47·5	18·69	8·68	
	2	F + irradiation	5·3	25·4	5·3	44·9	21·06	9·75	
	3	F + C.L.O.	5·3	28·0	6·4	39·3	21·60	9·92	
12	6	F	6·5	30·3	10·0	45·4	16·38	7·59	2·16
	6	F + C.L.O.	4·9	32·5	8·2	39·2	21·13	9·71	2·18
13	4	F	7·0	27·3	7·6	46·5	15·95	7·42	2·15
	2	F + C.L.O.	5·4	34·0	7·8	39·6	20·48	9·45	2·17
14	4	F_1	4·6	29·5	5·9	41·5	19·73	9·18	2·15
	4	F_1 + C.L.O.	4·9	34·3	6·0	40·5	21·08	9·87	2·14
	4	F_1 + sodium phosphate injections	4·3	32·1	6·5	45·6	19·54	9·15	2·14
	4	F_1 sodium glycerophosphate injections	4·7	29·5	5·2	42·4	20·21	9·36	2·16
15	2	F	4·5	28·2	5·8	46·7	19·06	9·00	2·12
	2	F + butter fat	4·6	30·5	5·3	41·4	20·86		
	2	F + oxidised C.L.O.	4·4	31·3	5·7	39·1	21·32	9·84	2·17
17	2	F	3·8	24·0	5·4	54·4	16·91		
	2	F + unsaponifiable matter of C.L.O.	4·5	28·0	4·6	46·6	19·80		
8	2	E.W. + casein	5·9	30·1	6·3	39·3	17·12		
	2	Do + C.L.O.	5·7	35·0	7·1	39·7	18·61		
	2	Do + 5 g. spinach	5·8	26·9	7·2	41·0	19·86		
	2	Do + 5 g. cabbage	5·9	37·8	6·6	40·0	19·05		
11	4	F	8·6	27·6	4·9	—	15·3		
	3	F + 0·1 g. spinach	8·8	31·1	4·4	—	15·9		

Diet F_1 (Exp. 14) contained 0·54 % of calcium and 0·54 % of phosphorus but was otherwise the same as diet F.

Diet E.W. + casein (Exp. 8) contained 0·67 % of calcium and 0·73 % of phosphorus.

The calcium and phosphorus contents of the other diets are given in Table I.

In Exp. 16 the 10 cc. milk increased the calcium content of the diet by about 10 % and the phosphorus content by about 30 %.

described by Kay and Robison [1924]. Rats from the same litter and in most cases from the Lister stock were used and the males and females were divided as equally as possible between the groups. Three experiments were also made on guinea-pigs but were not very satisfactory and the results have been omitted from the Table. The animals were killed in fasting condition[1] by severing the carotid and jugular without anaesthetic and the blood was taken directly into 1 % trichloroacetic acid to stop the action of the phosphoric esterase in the blood. This precaution, however, introduced a new difficulty. The cloudiness which occasionally appears on addition of the ammonium molybdate solution in estimating phosphate by the Briggs method, became more frequent and intense. The cause was finally traced to the presence of protein cleavage products formed by the action of a pepsin-like enzyme in the blood. The hydrolysis occurs much more rapidly in 1 % trichloroacetic acid than when the blood is laked with water. We continued to use the 1 % trichloroacetic acid for laking but added the further quantity of the acid as soon as possible to precipitate the proteins. This difficulty was only encountered in the case of rat's blood. Human blood and that of rabbits appear to contain much less "pepsin." The results of these experiments are set out in Table II.

Inorganic phosphate. As in the previous series the concentration of inorganic phosphate in the blood of the rats on diet 3143 was abnormally low but was raised almost to normal by the addition of cod-liver oil. The figures for the groups receiving other diets containing a sufficiency of inorganic phosphate provide no evidence that the deficient calcification of the bones produced in absence of the fat-soluble factor is associated with an abnormally low concentration of inorganic phosphate in the blood or that this concentration is raised by cod-liver oil or other sources of the vitamin. The figures for the normal controls show considerable variations and are obviously affected by other factors of which we have insufficient knowledge.

Organic phosphoric esters. In the groups on diet 3143 the total amount of phosphoric esters was again not raised by cod-liver oil except in one experiment (18) in which the rats were exceptionally well grown, being older than usual when placed on the diet. On the other diets the total amount of phosphoric esters was increased by the addition of cod-liver oil, by oxidised cod-liver oil, by the unsaponifiable fraction of the oil and by cabbage. Against the conclusion that this increase is associated with the improved calcification of the bones must be placed the fact that in Exp. 3 the amount of the increase was vanishingly small although the improvement in the calcium content of the bones was very marked. On the other hand, in Exp. 11 a decided increase in the amount of the esters was accompanied by very slight improvement in the bones. Irradiation appeared to have little effect on this value.

As to the phosphoric ester which is hydrolysable by the bone enzyme, we can find from these figures no satisfactory evidence of any abnormally low concentration in the blood of rats on −A diets nor of any significant regular increase connected with the addition of cod-liver oil to the diet. Whether the increase in the amount of the other ester which is not hydrolysable by the bone enzyme may have any indirect effect on the calcification of the skeleton we are not at present prepared to offer an opinion. Possibly the investigation at present in progress of the phosphoric-esterase of the blood may throw some light on this question. The activity of this enzyme appears to be subject to very delicate control by the p_H of the blood and to influence in a very marked degree the inorganic phosphate level [Martland, 1925].

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[1] Except in Exp. 14, in which the rats were killed two hours after feeding and injections.

Effect of injecting sodium glycerophosphate.

As an additional method of discovering whether the defective calcification is due to a lack of the specific phosphoric ester it was further proposed to study the effect of injecting sodium glycerophosphate into rats on a diet deficient in the fat-soluble factor. Unfortunately, owing to the fact that the experiment (Exp. 14) was carried out during the summer months with rats from a particularly strongly growing stock, the controls failed to exhibit very marked symptoms and the percentage calcification of the bones was not greatly below that of the normal animals. Two litters of rats, divided into four groups each of four animals, were placed on the experimental diets when 21 days old, and 10 days later the injections of sodium glycerophosphate (1 cc. $M/5$ solution daily, subcutaneously) were commenced in one group. A second group received injections of equivalent amounts of sodium phosphate (Na_2HPO_4) while a third received no injections. The fourth group received cod-liver oil (by mouth) from the beginning to the end of the experiment which lasted 57 days. Analyses were made in duplicate of the bones of the hind legs of all animals of each group collectively and the blood was also investigated (Table II). The average Ca content of the dry bones of the groups receiving sodium glycerophosphate injections was 2·4 % higher than that of the groups receiving none and 3·4 % higher than that of the group receiving sodium phosphate injections which was even lower than that of the controls. Cod-liver oil increased the calcium content of the bones by 6·8 % above that of the $-A$ group. The evidence of this experiment, that the injection of sodium glycerophosphate produces some increase of calcification while that of sodium phosphate has no such effect, could not, in view of the small increases observed, be considered satisfactory without further confirmation. We did not, however, continue these experiments as the subject was being very thoroughly investigated by Korenchevsky and Carr [1925]. As the result of their experiments they conclude that sodium glycerophosphate has a definite effect in increasing the calcium content of the bones of such rachitic rats, while injections of sodium phosphate produce less or no improvement. They find however that calcium glycerophosphate produces a much greater improvement than the sodium salt.

If we may draw any conclusion from these results and from our own, it is that while a deficiency of the specific phosphoric ester is not the cause of the defective calcification, an excess of this ester may go some way towards counteracting the lack of the unknown responsible factor. The marked effect of calcium glycerophosphate suggests but does not prove that the deficiency is in the calcium ions.

That the sodium salt of the phosphoric ester possesses some beneficial effect not shown by sodium phosphate may be taken as additional evidence of the participation of the bone enzyme in the process of calcification.

The Ratio of Ca *and* P *in the bones.*

Both the phosphorus and calcium were determined in the bones of the experimental animals as it was thought possible that variations in the ratio Ca : P might throw light on the deficient factor. This ratio would be 1·93 for $Ca_3(PO_4)_2$, 2·15 for calcium carbonato-phosphate $(Ca_3P_2O_8)CaCO_3$ or for hydroxyapatite $(Ca_3P_2O_8)_3Ca(OH)_2$ (Bassett), and 2·37 for a mixture of 1 mol. hydroxyapatite with 1 mol. $CaCO_3$ (9 %). If the carbonate and phosphate are precipitated independently a deficiency of the phosphate ion might give rise to bones showing a higher ratio of Ca : P if the factors governing the precipitation of $CaCO_3$ were unaffected. A deficiency of the Ca ion would not necessarily alter the ratio, unless the calcium were replaced by magnesium. Calculations based on the published analyses did not furnish very hopeful results. Thus Gabriel's [1894] analyses give ratios of 2·27 for human bones, 2·24 for ox bone and 2·19 for goose bone; Gassmann's [1910] analyses of bones of normal and rachitic children give 2·24 and 2·16 respectively, the lower ratio in the rachitic bone being accounted for by an increase in Mg from 0·1 % to 0·64 % of the dry bone. Our own analyses were carried out in duplicate on aliquot portions of solutions obtained by igniting the bones of hind legs with nitric and sulphuric acid. The Ca was estimated by the method of Aron and Sebauer and P by that of Neumann. It would have been better had the bones first been extracted with alcohol and ether but it was found by actual experiment that the amount of P thus extracted does not represent more than 0·2 %–0·3 % of the total P of the bone so that the error introduced in the ratio Ca : P is very small. In Exps. 3 and 5 the Ca and P estimations were done on separate bones and the ratios are therefore of no significance.

In the experiments in which the basal diets, F and F_1, were not deficient in phosphate the ratios show a considerable degree of constancy, all lying close around 2·15 (± 1 %). There is a general tendency for the ratio to be slightly lower in groups on −A diet than in those receiving cod-liver oil. The ratios for the groups receiving McCollum's low phosphorus diet 3143 are decidedly higher (2·25) but are reduced to the normal figure by cod-liver oil. Without wishing to press these figures too far it may be said that they would accord with the supposition that in rats fed on diet 3143 the deficiency is chiefly one of phosphate ions while on diets deficient only in the fat-soluble factor it is the concentration of Ca ions which falls below the normal.

In such a complex problem as that of rickets we do not wish to suggest that our conclusions have necessarily any wider application than for the particular cases investigated, *i.e.* defective calcification produced in rats by diets deficient in the fat-soluble factor. It is hoped to extend the investigations to the blood of other animals and of children in rachitic conditions and some data have already been obtained in this direction, but the need of a closer study of the various forms of calcium in the blood seems to be even more clearly indicated.

We are greatly indebted to Dr M. Boas, Dr H. Chick and to Professor Drummond for allowing us to investigate the blood of the animals in certain of their experiments (Exps. 8, 11, 16, 17) and for permission to quote their analyses of the bones of these animals.

SUMMARY.

The blood of rats fed on diets containing a sufficiency of calcium and inorganic phosphate but deficient in the fat-soluble factor contains a normal amount of inorganic phosphate and of the phosphoric ester hydrolysable by the bone enzyme, and these are not increased by the addition of cod-liver oil to the diet.

The amount of the phosphoric ester resistant to the action of the bone enzyme is usually increased by cod-liver oil or other sources of the fat-soluble factor but there appears to be no direct connection between this increase and the improvement in the calcification of the bones.

The defective calcification of the skeleton resulting from such diets is probably not due to any deficiency of inorganic phosphate or of the specific phosphoric ester in the blood, or of the enzyme in the bone by which the ester is hydrolysed.

An excess of hydrolysable esters (caused by the injection of sodium glycerophosphate) may however partly counteract the lack of the unknown responsible factor and produce some improvement in the calcification of the bones.

In rats fed on a diet (McCollum 3143) very deficient in inorganic phosphate the concentrations of inorganic phosphate and of phosphoric esters in the blood are below the normal. The inorganic phosphate level is raised almost to the normal by cod-liver oil but the amounts of the esters are not significantly increased.

The ratios of Ca : P in the bones support the view that in rats fed on this diet the defective calcification is due mainly to a deficiency of inorganic phosphate ions while this is not the case when the defect is caused by a lack of the fat-soluble factor alone.

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XXII. AN ARITHMETICAL TEST OF THE VALIDITY OF THE THEORY OF BAYLISS REGARDING FERMENTATION AND ADSORPTION.

By JOHN BROWNLEE.

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Mt. Vernon, Hampstead, N.W. 3.

(*Received January 9th, 1925.*)

On page 315 or 316 in the various editions of Bayliss' *Principles of General Physiology*, a table is given, which is reproduced below, in which the values of the "specific activity" of the ferment trypsin when added in different concentrations to a solution containing equal quantities of caseinogen are shown, as determined by experiment.

Relative concentration of enzyme (1)	Time taken for equal change in minutes (2)	Mean rate × 1000 (3)	"Specific Activity" (4)	Theoretical values (5)
8	41	24	3	3
5	48	20·8	4·16	3·9
4	55	18·2	4·55	4·55
2	81	12·4	6·2	6·1
1	144	7	7	7

Bayliss remarks upon this table:

> "The reader will probably notice at once that these results are precisely what we should expect if the velocity were controlled by adsorption; it was, in fact, this relationship which first led me to suggest the hypothesis of adsorption as applying to the case."

Neither Bayliss, however, nor his present editors seem to have thought it worth while to test arithmetically whether the values obtained by the experiments are consistent with the usually adopted formula of adsorption.

Mathematically this is a piece of elementary algebra though the arithmetic is lengthy. The adsorption formula is usually written in the form,

$$x = \alpha C^{\frac{1}{n}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1),$$

where x is the amount of a specific substance adsorbed by a surface or surfaces, α a constant dependent on the units of measurement adopted and C the concentration of the specific substance remaining unabsorbed after equilibrium has been attained.

If the concentration of the substance, in the present instance trypsin, be originally m, this may be written in the form

$$x = \alpha (m - x)^{\frac{1}{n}} \quad \text{..............................(2).}$$

It is convenient, as $\frac{1}{n}$ is a fraction, to raise both sides to the power n. This is due to the fact that the unknown is contained in the expression on the right-hand side of the equation, not by itself but combined with a known but varying quantity m. The form of the equation used in calculation is thus

$$x^n = \alpha^n (m - x) \quad \text{..............................(3).}$$

If the amount of adsorption, as is assumed in Bayliss' theory, be taken as proportional to the rate of action, column (3) in the table, the equation can be easily solved. It is convenient for purposes of calculation to divide the figures in the third column of the table by seven making the lowest figure unity. With this notation, taking the first, third and fifth terms of column (3) and comparing with the corresponding amount of enzyme, column (1), we have the three equations

$$\left(\frac{24}{7} x\right)^n = \alpha^n \left(8 - \frac{24}{7} x\right) \quad \text{........................(4),}$$

$$\left(\frac{18{\cdot}2}{7} x\right)^n = \alpha^n \left(4 - \frac{18{\cdot}2}{7} x\right) \quad \text{........................(5),}$$

$$x^n = \alpha^n (1 - x) \quad \text{................................(6).}$$

The solution is obtained by dividing equations (4) and (5) by equation (6) and then eliminating x. The eliminant equation after reduction has the form

$$68{\cdot}6 \left(\frac{24}{7}\right)^n - 224 \left(\frac{18{\cdot}2}{7}\right)^n + 347{\cdot}2 = 0 \quad \text{.................(7).}$$

From this equation n has to be determined. This was solved by taking a series of values of n, namely, 2, 3, 4, and 5. The root having been located between $n = 4$ and $n = 5$, a series of approximations resulted in the final value of 4·1723. This gives $\frac{1}{n} = {\cdot}240$, a number in accord with all the values experimentally found to hold in adsorption experiments.

Using the values of n it was directly calculated that the value of α^n was equal to 22·84 and of x equal to ·973. From these the values of the "specific activity" for $m = 5$ and $m = 2$ were evaluated and are given in the fifth column of the Table. The chief difference which exists is that in the second line between the values 3·9 and 4·16. A second approximation distributing the error over all the observations would give a closer fit but as the labour of solving such equations is considerable, and as the fit is all that could be desired from the experiments, it does not seem worth the trouble. The calculation clearly demonstrates that with trypsin and caseinogen, the experiments are consistent with the theory that the action of a ferment is in accordance with the adsorption formula as generally accepted.

OBITUARY NOTICE.

HORACE TABBERER BROWN.

Born *July 20th, 1848.* Died *February 6th, 1925.*

H. T. Brown was the posthumous son of Benjamin Tabberer and he assumed the surname of his stepfather, Edwin Brown of Burton-on-Trent, a banker, and in his leisure hours a naturalist of considerable attainments, who, by his sympathy and encouragement, exerted a great influence on the life of his stepson. At the early age of 12 Brown became interested in science. At the time he was receiving a grammar school education he realised that in chemistry he had found his *métier* and he devoted most of his time to acquiring knowledge in this direction. His interest in chemistry was fostered by Böttinger and Griess, who were then in Burton. In 1865 Brown became a student at the Royal College of Chemistry at Oxford Street, Hofmann still being professor there. One of the junior assistants at the College was Cornelius O'Sullivan and a fellow-student of Brown's was his lifelong friend Dr H. E. Armstrong.

After two terms at the College of Chemistry Brown entered in 1866 the brewery of Worthington & Co. His first scientific work was concerned with analyses of the Burton waters and the treatment of crude sewage. At the age of 25 he became manager of the whole of the manufacturing department, a post he occupied until 1889 when he was appointed a managing-director. As there was but little time to spare for laboratory work Brown selected John Heron as his assistant. The first fruits of this combination was a re-examination of the whole question of starch and its transformation. This work on starch was continued for many years in collaboration successively with J. Heron, G. H. Morris, J. H. Millar and others. In all some nineteen papers were published on this fascinating and intricate subject.

In 1883 Brown took up the study of geology, being led to it by some pressing requirements in connection with the water supply of Burton. The result of this work, which entailed a good deal of field surveying, was embodied in a paper on the Permian Rocks of the Leicestershire Coalfield. He appeared to have felt that too much of his time was being spent over geology in view of the many other problems which suggested themselves to him. The malting process in those days was completely empirical and Brown turned his exclusive attention to the physiology and anatomy of germinating seeds and their bearing on industrial malting.

In 1890 he and Morris published in the *Journal of the Chemical Society* their classical work on "Researches on the Germination of the *Gramineae*," a paper which he regarded as his most important and original effort. The results of this work were far reaching and it affords a good example of the manner in which a careful study of an industrial process can react on pure science. In 1893 Brown found that hop strobiles contained a diastatic enzyme and this fact was the starting point of his work connected with the chemistry and physics of assimilation in green plants. In 1893, with Morris, a paper entitled "A Contribution to the Chemistry and Physiology of Foliage Leaves" was read before the Chemical Society, and this was the last work carried out at Burton.

Not long after his arrival in London Brown engaged J. H. Millar as his chemical assistant and the work on starch and its products of transformation was continued, the results being published in a well-known series of papers in the *Journal of the Chemical Society.* Brown also at this time, in conjunction with the late Spencer Pickering, investigated the thermo-chemistry of carbohydrate hydrolysis, which afforded interesting results. With F. Escombe he studied at the Jodrell Laboratory, Kew, some of the outstanding problems connected with the germination of barley. The work on the assimilation of atmospheric CO_2 by green leaves was continued and this led to a study of the phenomena of the diffusion of gases and liquids. The work was of great, if not of fundamental, importance, since it showed how it was possible to account for the sufficiently rapid ingress of atmospheric CO_2 necessary for the formation of carbohydrate on the assumption that the interchange of gases between the leaf and the outer air took place only through the stomatic openings and not through the whole area of the epidermis, a conclusion also reached by F. Blackman by the aid of a series of very beautiful experiments. They also attempted to determine the mode of interchange of energy between the leaf and its environment and an endeavour was made to ascertain what proportion of the solar radiant energy is actually utilised by the chloroplasts in carbohydrate production within the leaf. The results were embodied in the Bakerian Lecture for 1905.

In 1901 Brown established the Guinness Research Laboratory in Dublin for the purpose of studying some of the more important problems connected with the brewing business of that firm. Much of the purely scientific portion of the work was printed for private circulation. Between the years 1907 and 1913 three papers were published in the *Journal of the Institute of Brewing*, on the "Nitrogen Question in Brewing," some of which had important technical applications.

It is significant of Brown's remarkable energy and spirit of enquiry that during occasional visits extending over a number of years to a fruit and wine farm he acquired for one of his sons in Cape Colony, he introduced modern methods for increasing the yield of deciduous fruits by artificial cross-fertilisation of the trees. He also succeeded in improving the methods of wine-making at the Cape and published an account of his investigations on this subject in the *Journal of the Institute of Brewing* (1914).

In 1908 he was appointed a member of the Royal Commission on Whisky and Potable Spirits, and in 1909, in conjunction with the late Sir Edward Thorpe, he investigated the method of determining the original gravity in beers. This enquiry involved a great amount of work in a number of breweries in different parts of the United Kingdom and the final results were published in a series of reports. Corrected tables for the determination of original gravity were constructed and their use became official. In 1918 Brown read his last paper to the Chemical Society on his work on Diffusion.

From the years 1870 to 1918 Brown published 90 papers and memoirs, not a few of them being regarded as classics. The value of his work was recognised and his election as a Fellow of the Royal Society in 1889 was the forerunner of a number of subsequent distinctions. He was awarded the Longstaff Medal of the Chemical Society in 1894, a Royal Medal of the Royal Society in 1903 and the Copley Medal in 1920. For a time he was Treasurer and afterwards Foreign Secretary of the Chemical Society, and only failing health prevented him from accepting the Presidentship of that Society.

Julian L. Baker.

XXIII. THE DETERMINATION OF GUANIDINES IN URINE AS PICRATES

By JOHN SMITH SHARPE.

From the Institute of Physiology, Glasgow University.

(*Received December 4th, 1924.*)

Burns and Sharpe [1917] described a marked increase in the amount of guanidine and methyl-guanidine in the blood and urine of dogs in tetania parathyreopriva and in the urine in idiopathic tetany in children.

The method then used was based upon that of Koch and involved the use of mercuric chloride. It has been criticised by Greenwald [1924] on the ground that by the use of the mercuric salt creatine may be split into guanidine. Further, the precipitation with auric chloride is unsatisfactory as the salt is easily decomposed even by light; and, as the gold salt of guanidine is not very insoluble, there is a considerable loss. The objection that creatinine in the urine may in our investigations have been estimated along with the guanidine seems to be negatived by the finding of Burns [1917] that after administration of guanidine there is little if any increase of creatinine.

Later Findlay and Sharpe [1920], and Nattrass and Sharpe [1921] recorded the presence of methyl- or dimethyl-guanidine in the urine in two cases of adult tetany. The method used depends upon precipitation by tannic acid and subsequent precipitation of the bases with picric acid. By this method the objections to the process previously used are eliminated. The danger that creatinine, potassium and ammonium picrates may be precipitated along with guanidine picrate has to be considered, but the difference in solubility and character of the crystals obviates this danger.

This method has also been criticised by Greenwald on the ground that the precipitate may have in part consisted of creatinine and ammonium picrate [1924, p. 332].

Since the publication of the method in 1920, improvement in some particulars has been the object of study, and the modification now described has been devised.

Method.

250 or 500 cc. of urine were treated with a 10 % solution of tannic acid. This was added in small quantities at a time and shaken after each addition. The precipitant was added until the supernatant liquid appeared green in colour. The precipitate was allowed to settle and filtered off by suction. To the filtrate $Ba(OH)_2$ was added in excess, the precipitate of barium tannate

allowed to settle and filtered. To remove the excess of hydroxide a few cc. of a 1 % H_2SO_4 solution were added in slight excess; this excess was then neutralised by adding a cream of $BaCO_3$, testing with litmus. The filtrate from this was evaporated to a syrup which usually crystallised on cooling. When cool the mass was extracted thoroughly with absolute alcohol and filtered: about 60 cc. of alcohol were necessary for 250 cc. of the original urine. The alcohol was evaporated off and the mass again extracted with about 20 cc. of alcohol. All the guanidine bases, creatinine, traces of potash, soda and ammonia are in the extract. The alcohol was again evaporated off and the residue dissolved in 10 cc. water. A saturated aqueous solution of picric acid was now added until the total volume was about 20 cc. This was set aside to crystallise.

The following tests show the applicability of the method:

1. *Recrystallisation of mixed picrates.* 50 mg. creatinine hydrochloride and 50 mg. guanidine hydrochloride were dissolved in a few cc. of water and saturated picric acid was added to precipitate the bases. The precipitate was filtered and dissolved in about 20 cc. of water at about 80° and set aside to crystallise.

Recovery: 47·8 mg. (95·6 %) guanidine hydrochloride.

The creatinine picrate separated on further evaporation. Its presence, along with the guanidine or methyl-guanidine picrates may be recognised by Jaffe's test and its amount estimated by Folin's method.

2. *Recovery of guanidine from mixtures of urinary chlorides.* 50 mg. creatinine hydrochloride, 50 mg. guanidine hydrochloride, 100 mg. potassium chloride and 100 mg. sodium chloride were dissolved in a little water and the mixture was evaporated to dryness, as in the final stage of the process of extraction of bases. The crystalline residue was treated in the usual way with 15–20 cc. absolute alcohol, filtered, and the alcohol evaporated off. Guanidine and creatinine were retained in the filtrate along with a little potassium.

After evaporation 10 cc. of water were added, then 10 cc. of saturated picric acid solution. The guanidine was precipitated immediately. After the guanidine picrate had been separated by filtration further evaporation was allowed to occur and creatinine picrate was obtained. On still further evaporation to 10 cc. characteristic hexagonal needles of potassium picrate were obtained, but this was not estimated. A little more creatinine was obtained in this fraction.

Recovery: 43·8 mg. (87 %) guanidine hydrochloride and 27 mg. (43·4 %) creatinine hydrochloride.

Sodium picrate is so very soluble that it would be impossible to precipitate it in this way.

3. *Addition of guanidine and creatinine to normal urine.* 50 mg. creatinine hydrochloride and 50 mg. guanidine hydrochloride were dissolved in 250 cc. normal urine and the full process of extraction carried out. The creatinine was not estimated.

Recovery (in two similar experiments): 42·9 and 44·8 mg. (85·5 and 89·6 %) guanidine hydrochloride.

Should creatinine picrate appear along with the guanidine picrate it was found that a safe and reliable method of eliminating it was fractional crystallisation. The mixed picrates were heated with just sufficient water at about 80° to dissolve the less soluble guanidine salt; this was set aside and the guanidine picrate separated free from creatinine and potassium.

As an alternative to this method of crystallisation, the creatinine can be removed by adding pure blood charcoal in slightly acid solution. Folin and Denis [1916] claim that upwards of 90 % of the creatinine is thus eliminated.

Ammonium picrate is more soluble than the potassium salt and it comes down only in concentrated solutions. It is very easily identified by the deep colour of the crystals and by their very constant crystalline form.

SUMMARY.

1. A method for the estimation of guanidine and methyl-guanidine is described. The process is simple and rapid and the proportion of guanidine recovered is nearly 90 %, whilst the separation from creatinine and potassium is complete. The photographs (Plate I) should assist in the identification of the picrates found in the various fractions of the final stage.

2. As mercuric chloride is not used the possibility of conversion of other bases (creatine) to guanidine is eliminated.

My thanks are due to Professor Noël Paton for advice and help in carrying out this work. The expenses were defrayed by a grant from the Medical Research Council.

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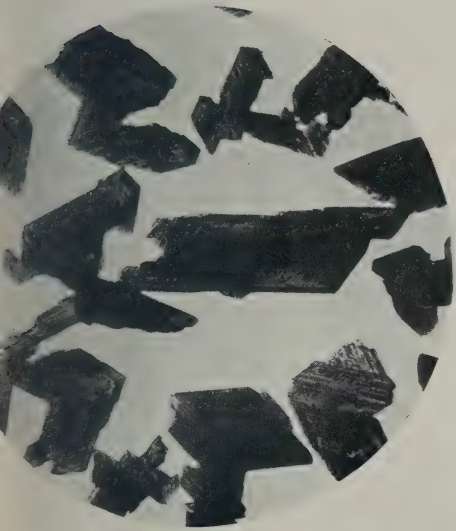
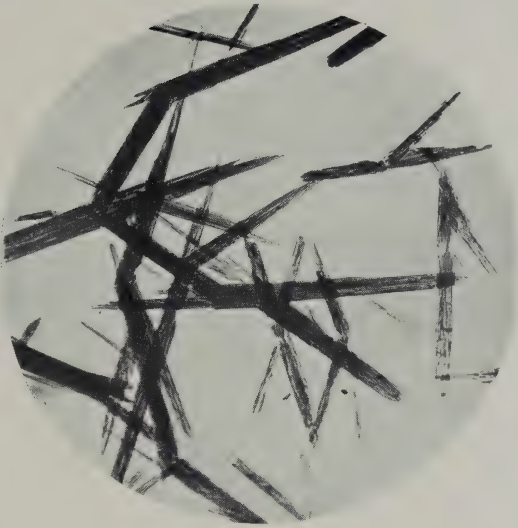
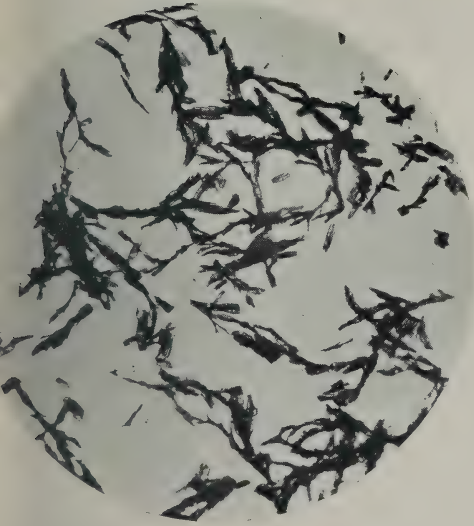


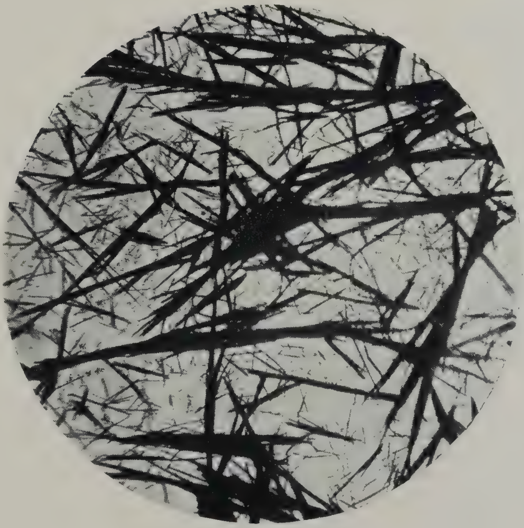
Guanidine picrate. × 150.



Methyl-guanidine picrate. × 150.



Dimethyl-guanidine picrate. × 150.



Creatinine picrate. × 150.

XXIV. THE DIAZO REACTION IN URAEMIC SERA.

By LESLIE FRANK HEWITT.

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(*Received January 12th, 1925.*)

The general concensus of opinion is that uraemia is due to the inability of the kidney to deal with certain normal or abnormal products of metabolism. Foster [1923] emphasises the selectivity of this retention by the kidney as contrasted with general retention of metabolic products (urinaemia). Thus, whilst in normal blood the residual nitrogen comprises some 10–20 % of the non-protein nitrogen present, in uraemic blood this value reaches 30–50 %. Attempts to identify the substance or substances responsible for the symptoms of uraemia have, so far, proved unsuccessful. Foster [1915] claims to have obtained from uraemic blood a material which caused the death of guinea-pigs; full experimental details have, however, not been published, and the amount of the material obtained and its properties are not given.

The diazo reaction may prove a valuable means of identifying at least one of the contributory factors in uraemia. Ehrlich [1882] and Pauly [1904, 1905] have described the red colour produced when a diazotised solution of sulphanilic acid is added to certain urines and the mixture rendered alkaline. Van den Bergh [1918] applied the diazo reaction to the detection and estimation of bilirubin in serum. When a dilute hydrochloric acid solution of diazotised sulphanilic acid is added to serum containing bilirubin a pink colour, turning green on addition of alkali, is obtained (*direct* reaction). In certain cases the reaction is obtained only when an alcoholic extract of serum is used (*indirect* reaction). This indirect reaction is conveniently conducted [McNee, 1923] as follows: 2 cc. of serum are poured into 4 cc. of 96 % alcohol, the coagulated proteins are filtered off and to 2 cc. of the clear filtrate are added 1 cc. of 96 % alcohol and $\frac{1}{2}$ cc. of dilute freshly-prepared *p*-sulphophenyldiazonium chloride.

Andrewes [1924], on applying this test to uraemic sera, observed the formation of a buff colour in place of the usual pink. This buff colour deepened gradually when allowed to stand for twenty-four hours, and when made alkaline with caustic soda this deepened buff colour changed to a bright rose red, which faded rapidly. Andrewes obtained this reaction with the sera from eight patients suffering from severe uraemia, but failed to obtain it with the serum of patients suffering from other diseases. This has been confirmed by the present author; the reaction has been obtained in the case of twelve uraemic patients[1] with blood urea concentrations of from 240 to 692 mg. per 100 cc. (mean value 366 mg.). As found by other workers [Van den Bergh, 1918; Andrewes, 1924; Feigl and Querner, 1919] these nephritic patients showed very marked hypo-bilirubinaemia[2]. In view of the possible connection between the substance causing the diazo reaction and that causing the symptoms of uraemia it was decided to investigate the matter further.

The conditions affecting the reaction itself were first studied. The conclusions reached were as follows:

(i) The characteristic of the reaction is the evanescent pink colour obtained on adding caustic soda, since the buff colour is more difficult to detect in pigmented sera and may be obtained with blood other than uraemic.

(ii) In place of the tedious waiting for twenty-four hours for the development of the buff colour, the test may be made without delay by boiling the reaction mixture for 30 seconds before the addition of caustic soda.

(iii) Strong bases (*e.g.* potassium hydroxide and baryta) produce the pink colour when added to the buff solution, whereas weaker bases (*e.g.* sodium carbonate and ammonia) do not.

(iv) The buff solution has been kept for as long as two months without apparent decomposition, whereas the pink coloration may fade in a few seconds, or at most lasts an hour, according to the intensity of the colour. When the pink colour has once faded the buff colour cannot be recovered by addition of acid, whereas the pink solution becomes buff on acidification and pink again on being rendered alkaline. Thus the fading of the pink colour indicates a reaction not reversed by acids.

(v) Sera do not themselves give the reaction, an alcoholic extract must be used.

(vi) Oxalated plasma may be used in place of serum and is often more convenient. The cerebro-spinal fluid, however, appears not to give the test. In one case of severe uraemia the urea in the C.S.F. reached the concentration of 692 mg. per 100 cc., but the test was negative.

(vii) The intensity of the reaction does not follow the variations in the concentration of urea in the blood, although in general the intensity of the reaction roughly follows the severity of the uraemia.

(viii) The presence of acetic acid interferes with the reaction and may even inhibit the appearance of the pink colour entirely.

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[1] The author is indebted to the following gentlemen for samples of uraemic blood: Dr G. A. Harrison, Dr R. D. Lawrence, Prof. Fraser, Dr Marrack and Dr Linder.

[2] Two infants of 4 and 6 months respectively with uraemia gave a negative test. This may possibly be due to a lower resistance than that of older patients, death therefore occurring at an earlier stage of the disease, before sufficient accumulation in the blood of the substance causing the reaction. It is noteworthy that these did *not* show hypo-bilirubinaemia.

EXPERIMENTS ON THE SUBSTANCE CAUSING THE REACTION.

(i) 24 cc. of an alcoholic extract of uraemic plasma (1 part of plasma to 2 of alcohol) were evaporated to dryness in an open dish; the residue was extracted with 2 cc. of absolute alcohol. Two drops of this extract were diluted to 1 cc. with a mixture of alcohol (2 parts) and water (1 part). This solution gave a strongly positive reaction with a diazotised solution of sulphanilic acid.

(ii) The substance is not removed from plasma or serum, when neutral, acidified or made alkaline, by ether.

(iii) 10 cc. of the alcoholic extract (66 % alcohol) were evaporated to one-third to remove the alcohol, made alkaline by the addition of a few drops of 40 % NaOH and extracted with 2 cc. of pure amyl alcohol. The aqueous layer showed a much diminished colour reaction, whilst the amyl alcohol gave a marked reaction. About six extractions of a deproteinised serum or plasma, made alkaline with either sodium hydroxide or sodium carbonate, with one-third the volume of amyl alcohol were sufficient to remove completely the substance causing the reaction. The amyl alcohol from such an extraction was removed by steam distillation, the material remaining in the aqueous residue.

(iv) The substance is entirely removed from an alcoholic extract by warming with a small quantity of absorbent vegetable charcoal and cannot be removed from the charcoal by warming with dilute acids or alkalis nor by extracting for ten hours with alcohol in a Soxhlet apparatus.

POSSIBLE IDENTITY OF THE SUBSTANCE CAUSING THE REACTION.

The substance possesses basic properties since it is extracted by amyl alcohol from alkaline solutions only. It is soluble in water, dilute acids and alkalis, in ethyl and amyl alcohols in the dilutions employed, but it is not soluble in ether. It is not volatile in steam and is stable to moderate heating. It is irreversibly adsorbed by charcoal. It cannot be detected in the blood of normal persons nor in that of patients suffering from diseases other than uraemia.

The simpler aliphatic amines are excluded on account of their volatility in steam and solubility in ether. Solutions of urea, creatinine and trimethylamine fail to give the reaction. It is probable that cyclic amines alone give this diazo reaction. In support of the hypothesis that the substance in uraemic blood causing the reaction is a toxic base containing the benzene nucleus or the iminazole grouping, *e.g.* tyramine or histamine, the following evidence can be adduced:

(1) Amines containing the phenyl or iminazole group give an evanescent pink coloration with an alkaline solution of diazotised sulphanilic acid [Koessler and Hanke, 1919, 1922].

(2) They are not volatile in steam [Barger, 1914].

(3) They are stable to moderate heating.

(4) They are not soluble in ether, but are extracted by amyl alcohol from alkaline solution [Koessler and Hanke, 1924, 1].

(5) Tyramine increases the blood pressure [Dale and Dixon, 1909], a

common symptom of uraemia; and tyramine produces renal disease and experimental nephritis in rabbits [Harvey, 1911].

(6) Koessler and Hanke [1924, 2] showed that over 60 % of the faeces they examined, both normal and pathological, contained bacteria which decarboxylated tyrosine and histidine to yield the corresponding toxic amines. Since these two amino acids are common products of digestion the presence in the body of the amines derived from them is readily explicable.

(7) Bain [1909, 1910] claims to have isolated tyramine from normal and pathological urines. Alcoholic extracts of urines frequently give (with the diazo reagent) a very similar reaction to that of uraemic blood.

(8) It has been shown [Koessler and Hanke, 1924, 3] that the liver plays a very subsidiary part in the detoxication of these amines, thus leaving the kidney to deal with them; so that, in case of kidney damage, such amines will not be readily eliminated from the body.

It would thus seem that the substance causing the diazo reaction in uraemic sera and plasma possesses many of the properties of the cyclic amines; further these amines are normally present in the intestine, and, as their elimination from the body depends on the kidney, nephritis would be expected to interfere seriously with their elimination. The accumulation of these amines in the blood in uraemia would thus be anticipated and may well account for the diazo reaction given by uraemic blood and for certain of the symptoms of uraemia.

SUMMARY.

(1) Andrewes' findings with regard to the diazo reaction in uraemic blood have been confirmed. It is possible to complete the reaction in one minute instead of twenty-four hours, by means of a slight modification.

(2) It is tentatively suggested that a cyclic amine, such as histamine or tyramine, may occur in uraemic blood and may cause the reaction.

The author wishes to express his deep indebtedness to Dr G. A. Harrison of the Hospital for Sick Children, Great Ormond Street, for providing the facilities whereby this work was carried out, and for his help and advice.

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XXV. STUDIES ON XANTHINE OXIDASE.

IV. RELATION OF XANTHINE OXIDASE AND SIMILAR OXIDISING SYSTEMS TO BACH'S OXYGENASE.

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In two recently published papers, one by Haas and Hill [1923], the other by Haas and Lee [1924], the authors state the existence in milk not only of a substance which can reduce nitrates but of another substance which, in the presence of aldehyde and molecular oxygen, can oxidise nitrites to nitrates. They call this latter substance itate, and explain its activity in the following way. In the presence of itate the aldehyde reacts with atmospheric oxygen to form an aldehyde peroxide, the oxygen of which is activated by the peroxidase of milk and oxidises the nitrite. Since the nitrate reducing power of milk has been shown by Dixon and Thurlow [1924] to be due, not to a special substance with that characteristic property, but to the xanthine oxidase plus any one of its substrates, the question arose as to whether the nitrite oxidising power of milk might not be ascribed to the same system. A possible mechanism which would bring about the oxidation of nitrites in such a system would be a coupled reaction. The substrate of the xanthine oxidase, having been activated by the enzyme, is oxidised by molecular oxygen. During the course of this reaction active oxygen would be produced and might oxidise the nitrite. If this were true, then not only aldehyde but hypoxanthine and xanthine in the presence of milk and molecular oxygen should bring about the oxidation of nitrites. The following typical experiment showed that such was indeed the case.

In each of four tubes were placed 10 cc. of milk and 0·2 mg. $NaNO_2$. To the first was added 1 mg. of hypoxanthine, to the second 1 mg. of xanthine, to the third 0·5 cc. of 10 % acetaldehyde. To the fourth no substrate was added. The tubes were aerated at room temperature for 45 minutes and then tested for nitrite by the addition of Griess-Ilosvay reagent, which gives a pink colour in the presence of nitrite. The intensity of this colour is proportional to the amount of nitrite present, and is indicated in the table by a varying number of + signs. A negative test therefore indicates that all the nitrite has been oxidised. The results are given in Table I.

If then, as this experiment indicates, the oxidation of nitrite is being in some way brought about by the ordinary oxidase of milk the reaction should occur upon the addition of any substrate of the enzyme, but not upon the addition of a substance not activated by the enzyme. The reaction should be inhibited by the same substances which Dixon and Thurlow have shown to inhibit the activity of the xanthine oxidase. Table I shows that in the presence of hypoxanthine, xanthine, aldehyde and adenine, all of which are activated by the oxidase of milk, nitrites are oxidised. The amount of nitrite oxidised is roughly proportional to the velocity with which the substrates are oxidised by the enzyme, as is shown in the table by comparison of the reduction-time of methylene blue by the various substrates. In the presence of those substances which are not activated by the oxidase, *e.g.* uric acid, guanine and caffeine, nitrites are not oxidised. Table I also shows that the oxidation of nitrites is inhibited by the same substances as inhibit the oxidation of hypoxanthine.

The effect of varying concentration of substrate was then investigated. Increasing the hypoxanthine concentration gave the inhibition expected from the results given in the previous papers, whereas increasing the aldehyde concentration increased the velocity of oxidation of nitrite, which result was also obtained in the previous work. An experiment typical of many is given in Table I. These results left little doubt that xanthine oxidase was a constituent of the system which was responsible for the oxidation of nitrites.

Table I.

Each tube in the aeration experiments contained 10 cc. milk, 0·05 mg. $NaNO_2$ and the substance given in column 1. In each case enough buffer of p_H 7·6 was added to keep the volume constant. In the anaerobic experiments each tube contained 5 cc. milk, 0·5 cc. 1/5000 methylene blue and the substance given in column 1.

Substance added	Time of aeration	Nitrite remaining unoxidised	Reduction time of methylene blue
Control	∞	+ + + +	∞
1 mg. hypoxanthine	45 mins.	–	2 mins.
1 mg. xanthine	45 ,,	+	4 ,,
0·5 cc. 10 % aldehyde	45 ,,	+ +	58 ,,
1 mg. adenine	8 hrs.	+ + +	50 ,,
1 mg. uric acid	8 ,,	+ + + +	∞
1 mg. guanine	8 ,,	+ + + +	∞
1 mg. caffeine	8 ,,	+ + + +	∞
1 mg. hypoxanthine	1 hr.	–	3 mins.
1 mg. hypoxanthine + 3 mg. uric acid	1 ,,	+ + +	15 ,,
1 mg. hypoxanthine + 2 mg. adenine	1 ,,	+ + + +	68 ,,
1 mg. hypoxanthine + 2 mg. guanine	1 ,,	+ + + +	15 ,,
1 mg. hypoxanthine + 2 mg. caffeine	1 ,,	+	3 ,,
0·6 mg. hypoxanthine	1 ,,	–	2 ,,
6 mg. hypoxanthine	1 ,,	+ + + +	16 ,,
0·2 cc. 10 % aldehyde	2 hrs.	+ + +	50 ,,
1 cc. 10 % aldehyde	2 ,,	–	10 ,,

If the oxidation of nitrites could be explained by the occurrence of a coupled reaction, the components of the system would be xanthine oxidase, substrate, nitrite and molecular oxygen. The system proved however not to be quite so simple, because, although whenever milk was used the nitrite was always oxidised, when the caseinogen-enzyme preparation described by Dixon and Thurlow was used, either with hypoxanthine or aldehyde, the nitrite was never oxidised. There must therefore be present in milk some necessary component of the system not present in the enzyme preparation. It was thought that this might be the fat, which is removed from the caseinogen-enzyme preparation by extraction with ether. Dixon and Thurlow have shown that fat accelerates the oxidation of hypoxanthine, and it was thought that the oxidation of nitrites might be so slowed up by the removal of fat that no change in nitrite concentration could be observed within the time of an ordinary experiment. Boiled cream was therefore added to the caseinogen-enzyme preparation, but again no oxidation of nitrite occurred. It was therefore obvious that the fat was not the required factor.

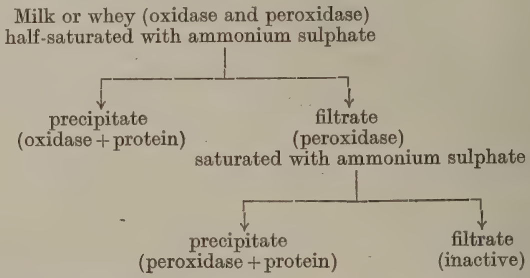
According to Haas and Lee when milk is coagulated with rennin and the whey half-saturated with ammonium sulphate, the precipitate obtained can, in the presence of aldehyde, oxidise nitrites, whereas the filtrate cannot. The filtrate would of course contain no oxidase, because this is precipitated by half-saturation with ammonium sulphate. According to the experiments already given showing that xanthine oxidase is connected with the nitrite reaction, a filtrate containing no oxidase could not be expected to oxidise nitrites. On the other hand when the precipitate was carefully washed with half-saturated ammonium sulphate until it was free from all traces of the filtrate, it behaved just like the enzyme preparation—oxidising hypoxanthine and aldehyde but not nitrites. The only difference between this procedure with the precipitate obtained by half-saturation with ammonium sulphate, and the procedure of Haas and Lee was the thorough washing. The obvious conclusion was that the filtrate contained the unknown nitrite-oxidising factor, which had been separated from the xanthine oxidase by the precipitation, so that neither precipitate nor filtrate caused the oxidation of nitrites. The recombination of the two should again complete the system, and give the same results as milk. This was actually found to be the case, as is shown in the following experiment. The filtrate containing the nitrite-oxidising factor was then saturated with ammonium sulphate and the factor was found to be precipitated completely. Repeating this experiment, using aldehyde instead of hypoxanthine, gives exactly the same results. Typical experiments are given below.

Each tube contained 0·05 mg. $NaNO_2$ + 0·4 mg. Hx + the substance given in column 1. The tubes were aerated at room temperature for 3 hrs.

Substance added	Nitrite remaining unoxidised
5 cc. half-saturated filtrate + 5 cc. 7·6 buffer	+ + + +
5 cc. precipitate obtained by half-saturation + 5 cc. filtrate	+
5 cc. precipitate obtained by half-saturation + 5 cc. buffer	+ + + +
5 cc. saturated filtrate + 5 cc. oxidase preparation	+ + + +
5 cc. precipitate obtained by saturation + 5 cc. oxidase preparation ...	−
5 cc. precipitate obtained by saturation + 5 cc. buffer	+ + + +
5 cc. oxidase preparation + 5 cc. buffer	+ + + +

It can be seen from these experiments that oxidation of nitrite occurs only when the nitrite-oxidising factor is added to the xanthine oxidase.

The question then arises as to the nature of this factor which, in the presence of molecular oxygen, xanthine oxidase and one of its substrates, can bring about the oxidation of nitrites. Haas and Lee consider the possibility of its being peroxidase, but discard it because the filtrate obtained from whey half-saturated with ammonium sulphate gives a peroxidase test but will not oxidise nitrites. It has just been shown that the combination of this filtrate and the precipitate obtained by half-saturation with ammonium sulphate oxidises nitrites, and that it is the addition of the peroxidase-containing filtrate to the xanthine oxidase which brings about the oxidation. The filtrate from whey or milk completely saturated with ammonium sulphate does not give the peroxidase test. Neither does it, when added to xanthine oxidase and hypoxanthine, cause the oxidation of nitrites. In no case has it been possible to get a solution which contained the milk peroxidase and which, when added to xanthine oxidase and its substrate, failed to oxidise nitrite. Nor, on the other hand, has it been possible to get a solution which did not give the peroxidase test but did contain the nitrite oxidising factor. Both the milk peroxidase and the nitrite-oxidising factor are destroyed by boiling but neither is destroyed by acid, so that it seems more than probable that the unknown factor is peroxidase. The following scheme makes clear the separation of xanthine oxidase from peroxidase.


Milk or whey (oxidase and peroxidase)
half-saturated with ammonium sulphate
precipitate
(oxidase + protein)
filtrate
(peroxidase)
saturated with ammonium sulphate
precipitate
(peroxidase + protein)
filtrate
(inactive)

It has not been possible to substitute a vegetable peroxidase in the system. Preparations of the peroxidases of horseradish, malt and potato were tried, but they all contain reducing substances which reduce nitrate with such alacrity that it is impossible to test for the disappearance of nitrite. The artificial peroxidases of Dony-Henault [1908] were tried. That made from ferrous sulphate reduced nitrates and was therefore of no use. The other, made from manganese formate, when substituted for milk peroxidase, did not bring about the oxidation of nitrites.

An animal peroxidase was then made in the following way. Ox liver was perfused until as much blood as possible had been washed away. It was then extracted with water and an equal volume of alcohol added to dissolve the remaining haemoglobin. The precipitate after repeated washing with 50 % alcohol was stirred up in water. The method of Kasanski [1912] for removing catalase by pyrogallol was tried, but it was found difficult afterwards to obtain the peroxidase free from pyrogallol, the presence of which renders impossible any comparison of pink colours obtained with the Griess-Ilosvay reagent. Owing to the presence of catalase it was impossible to get a negative test, *i.e.* to get all the nitrite oxidised, but in every case there was a distinct difference between the tube containing hypoxanthine and the control. An alkaline phosphate extract of washed ox muscle, which gave a faintly positive peroxidase test, was also tried a number of times. The results of these experiments are given below.

5 cc. liver peroxidase + 5 cc. oxidase preparation	+ 0·05 mg. $NaNO_2$	+ 0·3 mg. Hx	+ +
5 cc. liver peroxidase + 5 cc. oxidase preparation	,, ,,	—	+ +
5 cc. muscle extract + 5 cc. oxidase preparation	,, ,,	+ 0·3 mg. Hx	+ +
5 cc. muscle extract + 5 cc. oxidase preparation	,, ,,	—	+ + + +

These experiments show that an animal peroxidase can be substituted for the nitrite-oxidising factor and that therefore the latter must be peroxidase.

The next question to be considered is the rôle played by the peroxidase in bringing about the oxidation of nitrites in the presence of the xanthine oxidase and one of its substrates. Haas and Lee state that itate causes aldehyde in the presence of molecular oxygen to form aldehyde peroxide, the oxygen of which, when activated by the peroxidase, oxidises nitrite. They were able to get a test for peroxide when milk containing aldehyde was aerated. The activation of the aldehyde, however, as has been shown in the preceding experiments, is not due to an unknown factor, but to the milk oxidase, since any substrate of that enzyme can take the place of the aldehyde in bringing about the oxidation of nitrite. Therefore, if peroxide is formed it must be in the course of the oxidation brought about by the xanthine oxidase. The system would then be as follows:

1. Xanthine oxidase + substrate (Hx, X, or Aldehyde) + O_2 → peroxide
2. Peroxide + nitrite-oxidising factor (peroxidase) + nitrite → nitrate

Haas and Lee observed that nitrites were oxidised by hydrogen peroxide and milk peroxidase, but not by hydrogen peroxide alone. These experiments have been confirmed and the same results have been obtained with a system composed of milk peroxidase, nitrite and hydrogen peroxide as with a system composed of milk peroxidase, nitrite, xanthine oxidase and hypoxanthine or aldehyde. Since the second of the reactions given above is thus shown to occur, it remains to show that the first also occurs. Experiments were carried out to determine whether peroxide is formed in the course of oxidation of hypoxanthine, xanthine and aldehyde by molecular oxygen. Several tests for peroxide were used. Tincture of guaiacum was made up fresh each day and a water extract of horseradish scrapings, which had first been soaked in alcohol and dried, was added when the test for peroxide was made in order to supply the necessary peroxidase. Another very sensitive test is that suggested by Kaiser and McMaster [1908], a mixture of solutions of ferric chloride and potassium ferricyanide. Titanium sulphate was used in several experiments, but the yellow colour caused by the presence of peroxide was obscured to a large extent by the precipitated caseinogen so that it was less easy to detect small amounts of peroxide with this reagent than with guaiacum or the combination of ferric salts. The benzidine reagent could not be made to work in the presence of the caseinogen-enzyme preparation. A large number of experiments have been done in which a solution of caseinogen-enzyme preparation and hypoxanthine, xanthine or aldehyde has been aerated and tested at intervals for the presence of peroxide. Typical results are shown in Table II.

Table II.

In each experiment to 20 cc. caseinogen-enzyme preparation dissolved in p_H 7·6 phosphate buffer was added 1 cc. of substrate. The results are indicated by an arbitrary standard assumed for each test. The separate experiments are not comparable as far as the relation between time and amount of peroxide present is concerned, because, since various caseinogen-enzyme preparations were used, the activity of the enzyme was not the same in all cases.

Substrate	Test used	Time	Amount of peroxide present
Hypoxanthine	Guaiacum + horse-radish peroxidase	0	− (became blue on standing 10 mins.)
		10 mins.	+ + + +
		20 ,,	+ + + +
		40 ,,	+
		120 ,,	−
Adding more hypoxanthine		20 ,, after addition	+
Hypoxanthine	$FeCl_3 + K_3Fe(CN)_6$	0	−
		20 ,,	+
		60 ,,	+ +
		90 ,,	+
Hypoxanthine	Titanium sulphate	0	− (became faintly yellow on standing 15 mins.)
		20 ,,	+
		60 ,,	+
		90 ,,	−
Xanthine	Guaiacum + horse-radish peroxidase	0	− (became blue on standing 10 mins.)
		10 ,,	+ + +
		20 ,,	+ +
		40 ,,	< +
		60 ,,	−
Adding more xanthine		20 ,, after addition	+
Xanthine	$FeCl_3 + K_3Fe(CN)_6$	0	− (became blue on standing 20 mins.)
		20 ,,	+
		40 ,,	+
		60 ,,	+
Aldehyde	Guaiacum + horse-radish peroxidase	0	−
		15 ,,	< +
		30 ,,	+ +
		45 ,,	+ +
		60 ,,	−

These results have been obtained so many times that there seems to be no doubt that peroxide is formed in the oxidation of hypoxanthine, xanthine and aldehyde in the presence of molecular oxygen. The fact that the test is negative at the end of the reaction shows that the peroxide must be used up during the course of the reaction. This is explained by the observation of Wieland [1921] that hydrogen peroxide and various other peroxides act as hydrogen acceptors for activated hydrogen, forming water.

The destruction of peroxidase by peroxide was observed by Schellhase [1908]. If peroxide were being formed, one would expect to find the peroxidase disappearing as oxidation occurred. The gradual destruction of the peroxidase during the course of oxidation of aldehyde in molecular oxygen was observed by Haas and Lee and has been confirmed by my own experiments. I have found that peroxidase is also destroyed during the course of oxidation of hypoxanthine by molecular oxygen. The xanthine oxidase is also destroyed during the course of the reaction with molecular oxygen but not in the anaerobic oxidation.

Still further evidence for the belief that a peroxide is formed in the aerobic oxidation of hypoxanthine, xanthine and aldehyde, and that it is the activated oxygen of this peroxide which brings about the oxidation of nitrite, is afforded by the results obtained when these substances are oxidised anaerobically. In anaerobic oxidations methylene blue takes the place of molecular oxygen as hydrogen acceptor so that a peroxide could not be formed. According to the theory advanced earlier in the paper nitrite should not be oxidised. The following experiment shows that in the anaerobic oxidation of hypoxanthine the oxidation of nitrite does not occur.

Contents of vacuum tube	Reduction time of methylene blue
5 cc. diluted milk + 0·5 cc. methylene blue + 0·2 mg. Hx + 0·1 cc. H_2O	5·5 mins.
5 cc. diluted milk + 0·5 cc. methylene blue + 0·2 mg. Hx + 0·1 mg. $NaNO_2$	6 ,,

If the nitrite were oxidised, the reduction time of methylene blue should be decreased, whereas the reduction time with and without nitrite is the same.

Finally, the results obtained with catalase show definitely that hydrogen peroxide is formed. The work of various authors has shown that in a system containing both peroxidase and catalase the hydrogen peroxide present distributes itself between the two. Therefore if it is really hydrogen peroxide that is being utilised by the peroxidase to bring about the oxidation of nitrites, the reaction ought to be retarded by catalase. Catalase was made according to the method of Batelli and Stern [1904], and the solution of it used was adjusted to p_H 7·6.

Contents of tubes	Nitrite remaining unoxidised
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. catalase + 0·05 mg. $NaNO_2$ + 0·8 mg. Hx	+ +
3 cc. oxidase preparation + 3 cc. peroxidase + 4 cc. buffer p_H 7·6 + 0·05 mg. $NaNO_2$ + 0·8 mg. Hx	–

It can be seen that the reaction is inhibited to a marked degree by catalase. There seems to be no doubt therefore that in the course of aerobic oxidation of hypoxanthine, xanthine and aldehyde, hydrogen peroxide is formed.

The problem was now attacked from a different point of view. It has been shown that the oxidation of nitrite depends, not on the presence of peroxidase and a specific oxidisable substance like aldehyde, but on the presence of peroxidase and hydrogen peroxide. The hydrogen peroxide may be produced during the course of oxidation if the entire system is being considered, or it may be added to the peroxidase and nitrite. It might then be argued that if oxidising systems are at all comparable with one another it should be possible to substitute for the xanthine oxidase and its substrate other oxidising systems, such as another oxidising enzyme and its substrate, or a substance with a readily oxidisable group like the —SH compounds. Succinic oxidase and its substrate sodium succinate were substituted in the system for xanthine oxidase and hypoxanthine. The succinic oxidase was made by Ohlsson's [1921] method. The following is a typical experiment:

	Contents of tubes		Nitrite remaining unoxidised
5 cc. succinic oxidase + 5 cc. milk peroxidase	+ 0·05 mg. $NaNO_2$	+ 2·5 mg. sodium succinate	+
5 cc. succinic oxidase + 5 cc. 7·6 buffer	,, ,,	,, ,,	+ +
5 cc. succinic oxidase + 5 cc. milk peroxidase	,, ,,	—	+ + + +

This shows that succinic oxidase and sodium succinate behave like xanthine oxidase and hypoxanthine in the system. The succinic oxidase preparation gave a faint peroxidase test, which accounts for the fact that some nitrite is oxidised without the addition of milk peroxidase.

A solution of succinic oxidase and sodium succinate was aerated and tested without success for peroxide, probably because the peroxide is formed very much more slowly than in the case of hypoxanthine and is used as an oxidising agent as rapidly as it is formed. This is substantiated by the fact that the period of aeration of the succinic system must be about six times as long as that of the xanthine oxidase system with hypoxanthine before the same quantity of nitrite can be oxidised. The succinic oxidase preparation contains a large amount of catalase, and therefore no inhibition was obtained by the addition of more catalase. This result agrees with the curve obtained by Chodat and Pasmanik [1907] for the partition of hydrogen peroxide between peroxidase and catalase. They showed that the addition of catalase beyond a certain point caused no further inhibition of the peroxidase reaction.

The other type of oxidising system, namely that which does not require activation by an enzyme, was then studied. Xanthine oxidase and its substrate were replaced by cysteine, so that the system was now composed of cysteine, peroxidase and nitrite.

Contents of tubes	Nitrite remaining unoxidised
5 cc. cysteine + 5 cc. 7·6 buffer + 0·05 mg. $NaNO_2$	+ + + +
5 cc. peroxidase + 5 cc. buffer + 0·05 mg. $NaNO_2$	+ + + +
5 cc. cysteine + 5 cc. peroxidase + 0·05 mg. $NaNO_2$	–

Thus nitrite is oxidised when cysteine oxidises in the presence of peroxidase, and the inference can be made that during the oxidation of cysteine, peroxide is formed. Professor Hopkins (unpublished experiments) working with the titanium sulphate test was unable to show the presence of peroxide in oxidising cysteine solutions. This can be explained by the observation of Abderhalden and Wertheimer [1923] that cysteine is instantaneously oxidised by added hydrogen peroxide, so that if hydrogen peroxide were formed during the oxidation of cysteine it would be removed from the solution as soon as produced. Thus the quantity of peroxide in the solution at any instant would be too small to give a positive test. The effect of catalase was then tried but with a negative result; that is, catalase does not inhibit the oxidation of nitrites in the cysteine system. This might be considered as evidence for the formation of an organic peroxide by cysteine. On the other hand it may be explained by the assumption that the peroxidase acts upon very small quantities of hydrogen peroxide more rapidly than does catalase, so that the effect of catalase is so small as to be unobserved.

Further evidence for the formation of peroxide in the oxidising cysteine solution is the fact that during the oxidation peroxidase is destroyed.

Finally, glutathione was used instead of cysteine.

Contents of tubes	Nitrite remaining unoxidised
2·5 cc. glutathione (25 mg.) + 2·5 cc. 7·6 buffer + 0·025 mg. $NaNO_2$	+ + + +
2·5 cc. glutathione (25 mg.) + 2·5 cc. peroxidase + 0·025 mg. $NaNO_2$	+

The above experiment shows that the glutathione system is similar to the others that have already been considered, in that during the course of oxidation of glutathione in the presence of peroxidase nitrite is oxidised, and therefore a peroxide must be formed during the oxidation.

Discussion.

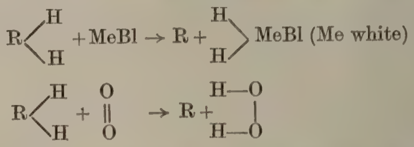
The fact that a peroxide is formed during the oxidation of hypoxanthine, xanthine and aldehyde has been established above, and since such systems as oxidising cysteine, oxidising glutathione and oxidising succinate behave in the same way in bringing about the oxidation of nitrite when peroxidase is added, there is little doubt that in these systems also peroxide is formed. The question arises as to the nature of the peroxide. Is it an organic peroxide or hydrogen peroxide? Bach [1913] believed in the formation of an organic peroxide in some oxidations and of hydrogen peroxide in others. His experimental evidence [1905] for the formation of an organic peroxide was the fact that when he added catalase to a system composed of plant oxygenase, peroxidase and pyrogallol, the oxidation of the pyrogallol was not inhibited. Therefore since catalase destroys hydrogen peroxide but not organic peroxides, that formed by the oxygenase must be organic. Bach [1905] showed, however, that pyrogallol destroys catalase so that in such a system no inhibition could be expected. There seems therefore to have been no evidence that an organic peroxide was formed during the course of such oxidations. Haas and Lee suggest that the peroxide formed in the oxidation of aldehyde is organic, but this view is not based on experimental evidence. There are strong reasons, however, for believing that the peroxide concerned is hydrogen peroxide. In the first place it seems unlikely chemically that compounds such as hypoxanthine would form peroxides. No compounds of such a type are known. Secondly, the explanation of the activity of the enzyme is far more simple if it is assumed that hydrogen peroxide is directly formed. The enzyme can then be considered as producing active hydrogen, a point of view which is generally assumed for enzymes of this type, and which makes the oxidation of hypoxanthine and aldehyde comparable to the oxidation of succinic acid. A general consideration of those systems which produce active hydrogen, and hence reduce methylene blue, shows that in every case the active hydrogen produced converts molecular oxygen to hydrogen peroxide [see Traube, 1893]. Armstrong and Colgate [1913] refer to a number of such systems, giving as typical examples:

1. The cathode in the electrolysis of dilute solutions of electrolytes. Any oxygen present is reduced to hydrogen peroxide by the active hydrogen produced.

2. When metals dissolve in acids, active hydrogen is produced which forms hydrogen peroxide with molecular oxygen.

3. Autoxidisable substances such as indigo white and hydrazobenzene during oxidation act as hydrogen donators, and the active hydrogen thus produced forms hydrogen peroxide with molecular oxygen.

Many other cases are known. In any system producing active hydrogen it would seem, then, that either of the following reactions might occur.



$$R{<}^{H}_{H} + \text{MeBl} \rightarrow R + {}^{H}_{H}{>}\text{MeBl (Me white)}$$

$$R{<}^{H}_{H} + \overset{O}{\underset{O}{\|}} \rightarrow R + \begin{array}{l}H-O\\ \qquad |\\ H-O\end{array}$$

Thirdly, as has already been mentioned, milk peroxidase acts upon hydrogen peroxide to bring about the oxidation of nitrites. Fourthly, that it is hydrogen peroxide which is connected with the nitrite oxidation in the system of oxidising hypoxanthine or aldehyde is shown by the inhibition by catalase. Bach and Chodat [1903] have shown that catalase does not decompose organic peroxides. Fifthly, Kastle and Loevenhart [1901] have shown that organic peroxides blue guaiacum directly without peroxidase, whereas hydrogen peroxide blues guaiacum only in the presence of peroxidase. A solution of oxidising hypoxanthine does not cause the faintest blueing of guaiacum until peroxidase is added, whereupon, as already stated, a deep blue appears. Sixthly, it has been shown that succinic acid oxidising to fumaric acid behaves in a manner exactly similar to oxidising hypoxanthine. A consideration of the formulae of succinic and fumaric acids makes the belief in the formation of an organic peroxide during this oxidation untenable; for while it is just possible to view hypoxanthine as a peroxide, it is inconceivable that succinic acid can do anything but lose two hydrogen atoms, which with molecular oxygen would form hydrogen peroxide.

The peroxides in the cysteine and glutathione systems remain for consideration. The reason for assuming the formation of hydrogen peroxide rather than of an organic peroxide in the case of the —SH compounds is only that such a view makes it possible to explain the mechanism of their oxidation in a manner more nearly comparable to that involved in the other oxidising systems which have been considered. It is impossible at the moment, however, as far as the —SH compounds are concerned, to make a definite assertion as to the nature of the peroxide formed. The case, however, for the direct formation of hydrogen peroxide rather than of an organic peroxide in the oxidation of hypoxanthine, xanthine, aldehyde and succinic acid seems well established.

We now come to a theoretical consideration of the results obtained. Bach maintained that a complete oxidising system consisted of two enzymes. The first was an easily oxidisable body which, with molecular oxygen, formed a peroxide and which he called oxygenase. The second was a peroxidase which acted upon the peroxide formed by the first enzyme, and the oxygen thus activated could bring about the oxidation of some other substance. Because of its properties, Bach believed the oxygenase to be an enzyme, although this point has been disputed by other workers. The Schardinger enzyme was, according to Bach, part of a quite different system. By means of water it reduced methylene blue in the presence of aldehyde, and was therefore called a reducing enzyme. As the same reactions have been shown by Morgan, Stewart and Hopkins [1922] to occur with molecular oxygen as acceptor as with methylene blue, the Schardinger enzyme, or rather aldehyde-oxidising enzyme (since in milk, as has been shown by Dixon and Thurlow, it is probably the same as the xanthine oxidase), can be considered as an oxidising enzyme. The Schardinger enzyme + aldehyde could then be considered as Bach's oxygenase except that the substance upon which the enzyme acts and not the enzyme itself would form the peroxide. Such a view would simplify the consideration of oxidations, as Bach's hydrolytic oxidation-reduction system could be thought of as comparable to his ordinary oxidising system. The experimental facts already given completely bear out such a conception, and explain why in some cases oxygenase seemed to behave like an enzyme, while in others it did not. Considering the oxygenase firstly from Bach's point of view that it is an easily oxidisable substance forming a peroxide, I have shown that in the oxidation of cysteine and glutathione, both easily oxidisable substances, a peroxide must be formed, because when the second part of Bach's system is added, namely peroxidase and another oxidisable substance, oxidation of the latter occurs. Cysteine and glutathione are therefore oxygenases, but cannot be considered as enzymes. But Bach's observation that in many cases the oxygenase is thermolabile and therefore behaves like an enzyme can be explained by the fact that oxygenase may not always be a single substance. In some systems it may be an oxidising enzyme and its substrate such as the xanthine oxidase and hypoxanthine, the aldehyde enzyme and aldehyde, or the succinic enzyme and succinic acid. I have shown that all these systems form peroxide, the oxygen of which in the presence of peroxidase can be used for further oxidations. Onslow [1920] has shown that in plants a catechol derivative and an oxidising enzyme form an oxygenase. She, however, refers to the enzyme as oxygenase, whereas by Bach's definition the term oxygenase includes both enzyme and peroxide-forming substance.

The only difference in the oxidising systems which I have shown to be oxygenases is that in some cases the oxidation consists of a loss of hydrogen and in others of an addition of oxygen. In the latter case it is necessary to suppose water as a reacting part of the system. The essential mechanism involved, however, can be assumed on Wieland's theory of hydrogen activation to be the same in all cases. Morgan, Stewart and Hopkins suggest that hypoxanthine adds on water to form a hydrate, the hydrogen of which is activated in Wieland's sense by the enzyme and is transported, if the oxidation be anaerobic, to methylene blue, or, if the oxidation be aerobic, to molecular oxygen. I have shown that in the latter case hydrogen peroxide is formed. It might be assumed that the oxidation of —SH compounds is similar to the oxidation of the hypoxanthine hydrate. Warburg claims that —SH compounds can oxidise only in the presence of iron. The iron might be considered as activating the —SH group, thus rendering its hydrogen labile. Mr D. C. Harrison (unpublished experiments) has recently obtained some results which make this view highly probable. The activated hydrogen would reduce methylene blue or with molecular oxygen would form hydrogen peroxide. The action of iron on the —SH compounds would on such a view be analogous to that of the oxidising enzymes on their substrates; and the conception of oxidising systems would be somewhat simpler.

SUMMARY.

1. Hypoxanthine, xanthine and adenine can replace aldehyde in the nitrite-oxidising system in milk, termed itate by Haas and Hill.

2. The caseinogen preparation of xanthine oxidase + one of its substrates will not bring about the oxidation of added nitrite, but will do so if milk peroxidase is added. The complete system consists of xanthine oxidase; hypoxanthine, xanthine or aldehyde; milk peroxidase and nitrite.

3. The peroxidase of liver has been substituted for the milk peroxidase in the nitrite-oxidising system.

4. Peroxide is formed in considerable quantities when hypoxanthine, xanthine or aldehyde is oxidised by the xanthine oxidase in the presence of molecular oxygen.

5. The oxidation of nitrite in the presence of milk, hypoxanthine and molecular oxygen is retarded by catalase.

6. Nitrite is oxidised by the system succinic oxidase, succinic acid, animal peroxidase and molecular oxygen.

7. Nitrite is also oxidised during the autoxidation of the —SH compounds cysteine and glutathione, provided milk peroxidase is present.

8. These results lead to the conclusion that peroxide is formed during the aerobic oxidation of succinic acid, cysteine and glutathione.

9. In the cases of hypoxanthine, xanthine, aldehyde and succinic acid hydrogen peroxide is formed directly by the addition of active hydrogen to the oxygen molecule.

10. The systems xanthine oxidase + any one of its substrates, succinic oxidase + succinic acid, cysteine and glutathione are shown to be oxygenases as defined by Bach.

11. A preparation of animal peroxidase suitable for general use can be obtained by saturating with ammonium sulphate the filtrate obtained by half-saturating milk with ammonium sulphate.

The author wishes to express her sincere thanks to Sir F. G. Hopkins for his continued interest and encouragement during the course of this work.

She is also extremely grateful to Mr M. Dixon for much helpful advice and invaluable criticism.

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XXVI. STUDIES ON THE NATURE OF THE PROCESS OF GERMINATION.

A NEW METHOD FOR THE DETERMINATION OF PROTEINS BY MEANS OF ADSORPTION APPLIED TO THE DECOMPOSITION OF PROTEINS IN GERMINATING PEA SEEDS.

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(*Received January 19th, 1924.*)

Earlier publications dealing with the question of decomposition of proteins in germinating seeds, have not given adequate consideration to proteolytic hydrolysis accompanying the extraction process.

Osborne has pointed out that a short extraction at a normal temperature, even if incomplete, yields more satisfactory results than prolonged extraction with heat because in the latter case it is impossible to avoid changes resulting from fermentation.

It is for this reason that the figures given in earlier publications relating to the proportion of protein N to amino N require to be reconsidered. As we are dealing only with proteins which are soluble in neutral solutions of sodium chloride (globulins and albumins) it will be best not to attempt a complete extraction but to determine the proportion of protein nitrogen to amino nitrogen in equivalent parts of the maceration liquid.

It will be later shown that it may be possible to obtain approximately absolute figures.

The usual methods for the determination of proteins were also considered to be unsatisfactory.

The method most often applied in Agricultural Chemistry, *i.e.* the determination of the total nitrogen content and multiplication by a certain factor, is of course useless in this case, in which proteins as well as non-proteins (amino acids, etc.) have to be taken into account.

Precipitation with phosphotungstic acid [Abderhalden and Pettibone, 1912] cannot furnish exact data for the degree of protein decomposition. This precipitation process is accompanied by the precipitation of other more or less complicated protein derivatives together with the protein itself.

Similarly the "Stutzer" method [Rosenthaler] (precipitation with copper hydroxide) does not yield accurate results.

Osborne's method [1924] of fractional "salting-out" is, apart from its intrinsic difficulty, quite useless for our purpose, because it fails completely as a quantitative method. (The method was in fact never worked out for this purpose.)

With a view to separating the proteins from other nitrogenous substances, we have tried to adsorb them by suitable adsorption media and afterwards to eluate[1] them.

Adsorption with aluminium hydroxide gel was first tried. As this substance, except in the presence of special reagents which had to be avoided, is easily soluble in excess of alkali, the less soluble iron hydroxide gel was chosen. It was proved that relatively small quantities of iron hydroxide gel were quite sufficient to adsorb quantitatively all the proteins. The filtrates from this adsorption did not react in the least to Spiegler's or Esbach's reagent, *i.e.* they were absolutely free of proteins.

By the use of weak alkalies it is possible to eluate the proteins almost quantitatively.

An experiment with kaolin (the purified kaolin of May and Baker was used) failed, at first, completely; better results were secured only after slight acidification, *i.e.* acidification of the maceration liquor in slight excess of the optimum for the coagulation of the proteins.

A maceration liquor showed, for example, a p_H of 4·8 (determined with platinum coated electrodes by Luther's method), and was adsorbed only to a slight extent at that concentration. Only when the p_H had been brought to the value of 3·8 by acidification was the adsorption brought practically to completion. The filtrates showed only a weak protein reaction with Spiegler's reagent, after standing for a long time. From the kaolin adsorbate it was possible to eluate the proteins almost quantitatively.

The following figures indicate comparative results with iron hydroxide gel and with kaolin.

100 cc. of maceration liquor contained:

	$Fe(OH)_3$	Kaolin
Total N before adsorption	0·451	0·451
Non-protein N after adsorption	0·038	0·099
Protein N	0·413	0·352
First elution (2 % NaOH) N	0·280	0·288
Second elution (4 % NaOH) N	0·093	0·034
Third elution (6 % NaOH) N	0·020	0·009
Elution (1+2+3) N	0·393	0·331

It is evident that it is possible to eluate the protein N almost quantitatively. The higher content of non-protein N in the filtrate from the kaolin adsorption is easily explained, because as we have already mentioned the first adsorption by kaolin is by no means absolutely quantitative.

Previous experiments [Abderhalden and Fodor, 1919] and our own experience show that no amino acids are adsorbed when using such reagents.

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[1] We are adopting the terminology of Willstätter.

The method of determination is briefly as follows: 100 g. of finely-ground seed-flour is macerated for one hour with 500 cc. of a solution of 10 % sodium chloride at the temperature of the room.

The liquor is then filtered through a battery of folded filter papers and toluene and thymol are added for preservation. In this maceration liquor the nitrogen content is determined by Kjeldahl's process (micro-method of Abderhalden-Fodor).

100 cc. of the maceration liquor is mixed with a corresponding quantity of iron hydroxide gel, completely neutralised and well shaken for half-an-hour. (The iron hydroxide gel was prepared by precipitation of a solution of $FeCl_3$ with the equivalent amount of sodium carbonate and removal of excess CO_2 by boiling.) The resultant precipitate is filtered and thoroughly washed. The filtrate must be absolutely free from proteins; if this is not the case the quantity of iron hydroxide used has been insufficient. The non-protein nitrogen in the filtrate is then determined. To recover the proteins, the residuum is eluated for 2 hours at first with 2 % NaOH, subsequently with 4 % NaOH and finally with 6 % NaOH. About 300 cc. of alkaline liquor are used for both elution and washing on each occasion. Finally by acidifying up to the optimum of coagulation, the proportion of protein which can be precipitated and the proportion non-precipitable can be determined. Protein which cannot be precipitated by acid can be dialysed and isolated. The difference between the amount of total N in the maceration liquor and the adsorption filtrate indicates the content of protein nitrogen. Lipins are present in such small quantities that they can be ignored.

To obtain approximately absolute figures, it is only necessary to determine the proportion of nitrogen in the flour and the nitrogen content in the maceration liquor and to multiply the protein nitrogen content found in the maceration liquid with the corresponding factor.

Thus we found a total nitrogen content of 0·451 % in the maceration liquid, whereas it contained 0·413 % of protein nitrogen. The nitrogen of the seed-flour used amounts to 3·976 %.

It was therefore necessary to multiply the figure obtained by the factor $\frac{3{\cdot}976}{0{\cdot}451} = 8{\cdot}82$. From this, by multiplying the amount of protein nitrogen by 5·7 (the usual factor for conversion of the nitrogen of Leguminosae into protein) [Grafe, 1914], the protein content is found to amount to 20·76 %.

The alternative method of complete extraction cannot be recommended, because as we have already mentioned, proteolytic hydrolysis is liable to take place under such circumstances.

In the examination of germinated seeds smaller quantities of elution reagent and a shorter period of reaction have been used although we did not recover all the proteins in this way.

This new method of protein determination in plant extracts possesses in our opinion the following advantages:

(1) The proportion of protein nitrogen to non-protein nitrogen is quantitatively determined.

(2) The proteins are purified and can easily be recovered.

(3) Proteolytic hydrolysis is avoided.

(4) The method has the advantage of great rapidity.

EXPERIMENTAL.

The peas, "Prince Albert," supplied by the firm Vilmorin, which contain 3·976 % of nitrogen (air dried after removal of the skin) were used. They were sown in germination boxes and Tel-Aviv dune-sand was used as substratum.

The following summary explains the plan of our experiment:

	Root		Stalk
Experiment Light 1 (after 3 days)	3–4 cm.		—
Experiment Light 2 (after 11 days)	Strongly developed	*Ca.* 5 cm.	Cotyledons green
Experiment Dark 1 (after 3 days)	3–4 cm.		—
Experiment Dark 2 (after 11 days)	Strongly developed	*Ca.* 8 cm.	Cotyledons pale

As we had to work on the problem of chemical processes in the seed itself, the latter had to be completely separated from skins, roots and stalks. The following figures indicate the content of protein N and non-protein N.

In 100 parts of the maceration liquor:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Total N	0·451	0·560	0·648	0·526	0·671
Non-protein N	0·038	0·058	0·143	0·065	0·139
Protein N	0·413	0·502	0·505	0·461	0·532

From these figures the proportion of protein N to non-protein N can easily be calculated.

100 parts of N contain:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein N	91·57	89·64	77·93	87·62	79·28
Non-protein N	8·43	10·36	22·07	12·38	20·72

This summary shows us that there is a steadily increasing dissociation which has about the same value for normal and for etiolated germinated seeds for equal intervals of time. Previous literature has generally given a figure of 89 : 11 for the proportion protein N : amino N [Adolf Mayer].

This somewhat high content can probably be explained by the fact that proteolytic hydrolysis had occurred during the extraction process.

The following figures of Schulze and Flechsig [1885] for germinated seeds can serve for comparison (the peas were germinated on saw-dust in the dark and the figures relate to the whole plant).

N in % of total N:

	Not germinated	First stage
Protein N	86·44	38·33
Amino N	11·62	30·34
N soluble in ethyl acetate	1·94	11·33

The higher content of amino acids in the first stage may be chiefly ascribed to the fact that all these analyses relate to the whole plant. It is well known, *e.g.*, that the formation of asparagine is chiefly located in the axial portions of the plant.

Schulze [1878] found in 11-day old lupins in the axial portions 31·81 % asparagine in the cotyledons, only 7·62 % in the dry substance. Priaschnikow obtained similar results. It is confirmed that with regard to the axial portions, the N of the asparagine forms a much higher percentage of the total N than in the cotyledons.

Our experiments showed that not all the protein derivatives are immediately utilised for regeneration, but that a high percentage remains in the seed. Later experiments will enable us to decide whether after the dissociation of protein there is also a qualitative difference between the amino acids immediately used for regeneration and those which at first remain in the seed.

Osborne separated the proteins contained in peas by means of fractional "salting out" in different concentrations. We preferred to distinguish between protein fractions, which can be precipitated and those which cannot be precipitated by acid.

We therefore determined on the first two elutions the proportion between these two fractions. For this purpose we carefully acidified to the optimum of coagulation (*i.e.* methyl red showed a weak reaction), and obtained the following results, expressed as % of protein N in the first two elutions:

	Not germinated	Light 1	Light 2	Dark 1	Dark 2
Protein precipitable by acid	31·17	44·02	47·14	47·08	57·75
Protein non-precipitable by acid	68·83	55·98	52·86	52·92	42·25

These figures are in no way quantitative, as the proportion was only determined in two elutions, in addition to which, as already mentioned, complete recovery of proteins was not carried out, because of the danger of proteolytic changes. It may be mentioned for the characterisation of these proteins precipitated by acid that *they are quantitatively dispersed in alcohol of* 70–80 %.

We hope to make further investigations of the alcohol sols of the plant globulins.

As it has been suspected that these proteins contain phosphoric fractions [Osborne and Strauss, 1915] this question was also examined. According to our experiments iron hydroxide gel is not suited for this purpose as it liberates the phosphoric acid on its surface.

We therefore left the protein of our maceration liquor to be adsorbed by kaolin and examined it after removal of all the proteins to see whether a difference could be observed in the content of phosphoric acid before and after the adsorption.

		% P	
	Non-germinated	Light 1	Dark 1
Maceration liquor before adsorption	0·07	0·06	0·05
Maceration liquor after adsorption	0·07	0·06	0·04

Hence it follows that there are no phosphoric fractions either in the ungerminated or in the germinated seeds.

SUMMARY.

(1) The method of adsorption with iron hydroxide gel is very well suited for the determination of protein and the derivatives of the protein decomposition process. It enables us to obtain the proteins in a pure state and to characterise them.

(2) The content of non-protein N in germinating seeds increases almost equally during the process of germination, whether the germination takes place in dark or in light.

(3) Only a part of the derivatives obtained at the cost of the proteins is immediately used for regeneration.

(4) In non-germinating seeds there is a proportion of 30 : 70 of protein precipitable by acid to protein not precipitable by acid; this proportion changes in germinating seeds where we find almost equivalent quantities of the two kinds of protein. (At the same time there is a loss of total protein N.)

(5) The protein precipitated by acid is dispersed by alcohol of 70–80 %.

(6) Neither in non-germinating seeds nor during germination are there any phosphoproteins.

We have to thank Miss S. Beham for her help in the analytical part of the work.

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XXVII. GLUTATHIONE.

THE OCCURRENCE AND QUANTITATIVE ESTIMATION OF GLUTATHIONE IN TISSUES.

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(Received January 21st, 1925.)

The sole information available as to the glutathione content of various tissues is based upon the amounts actually isolated as described by Hopkins [1921] in his original paper.

The figures are as follows:

Yeast. 0·1–0·15 g. per kg.; 0·01–0·015 %.

Muscle. Approximately the same.

Liver. Undoubtedly richer, but yield not recorded.

Professor Hopkins informs me that from yeast later yields of the dipeptide have been greater. In one case 14 g. were isolated from 50 kg. of yeast, a yield of 0·03 % approximately.

As the method of isolation is such that fairly considerable losses are bound to occur, it is obvious that the above figures are of the right order only.

An attempt was therefore made to estimate, more directly and quantitatively, the amount of glutathione in various animal tissues.

Experimental.

The method adopted was as follows. A known weight of the tissue is ground in a mortar with sand and 10 % trichloroacetic acid. The mass so obtained is filtered on a small Buchner funnel and re-extracted twice with further amounts of 10 % trichloroacetic acid.

It was found by the application of the nitroprusside test that this was sufficient to extract all the soluble —SH groups of the tissues.

The use of trichloroacetic acid has the following advantages:

(i) A clear protein-free filtrate is obtained.

(ii) It does not interfere with the subsequent iodine titration.

(iii) Oxidation of the —SH groups during the extraction is prevented owing to the acidity of the solution.

(iv) Glutathione is of course not precipitated by this reagent.

The clear extracts so obtained are titrated with *N*/100 iodine solution, using sodium nitroprusside as an external indicator. From the equation

$$2\mathrm{R.SH} + \mathrm{I}_2 = \mathrm{R.S.S.R} + 2\mathrm{HI}$$
$$1 \text{ cc. } N/100\ \mathrm{I}_2 \equiv 2{\cdot}5 \text{ mg. reduced glutathione.}$$

The method has been controlled as far as possible in the following ways. The following substances, which might be present in an extract of tissue prepared as described and which might also react with iodine under such conditions, have been found not to interfere with the estimation:

Urea, Uric Acid, Creatinine.
Glucose. Fructose.

Known amounts of reduced glutathione added to the tissue pulp were satisfactorily estimated.

Estimations have also been made of the sulphate sulphur and total sulphur contained in tissue extracts. The difference gives the soluble organic sulphur. This amount has been found to correspond well with the value obtained for the sulphur present as —SH groups by the iodine titration method described. In no case has the sulphur calculated from the iodine titration been greater than the amount of total organic sulphur.

The following table gives some typical results.

Extract	Iodine titration; cc. $N/100\ \mathrm{I}_2$	Sulphur calculated from iodine titration as mg. BaSO_4	Total sulphur mg. BaSO_4	Sulphate sulphur mg. BaSO_4	Organic sulphur mg. BaSO_4
Liver (rabbit)	15·3	35·6	37·3	0·7	36·6
Liver (rabbit)	10·3	24·1	32	5	27
Yeast	9·6	22·4	30	3	27

It follows, therefore, that by the iodine titration of the trichloroacetic acid extract of the tissue, the —SH groups and the —SH groups alone are being estimated. The results obtained by the method are given in the table following. In calculating, the assumption has been made that all the —SH groups present are those of glutathione. This assumption is considered further in a later section.

The following procedure was adopted with animals. They were chloroformed or stunned, and bled to death from the jugular vein. The tissue required was removed as rapidly as possible and weighed. In the case of muscle, preliminary chopping with scissors before grinding with sand, was found advantageous. Cellular organs (liver and kidney) were ground directly with sand.

In the case of yeast the cells were ground with acid, transferred to a beaker and warmed to 60°, and allowed to cool before filtering. Kieselguhr was used if difficulty was experienced in obtaining a clear filtrate.

Tissue	Weight (g.)	cc. $N/100$ I_2	mg. reduced glutathione	%	Remarks
Yeast. A (1)	20	13·6	34·0	0·17	Fresh baker's yeast.
(2)	10	7·2	18·0	0·18	
(3)	5	4·2	10·5	0·21	
(4)	5	3·8	9·5	0·19	
B (5)	5	6·0	15·0	0·30	Samples had dried through keeping.
(6)	5	5·6	14·0	0·28	
Rat. Liver. A (1) ...	8·0	6·08	15·2	0·19	Animals starved for 8 hrs. to free liver from glycogen.
(2) ...	7·8	5·6	14·0	0·18	
B (3) ...	7·5	4·8	12·0	0·16	
(4) ...	9·0	6·10	15·3	0·17	
Rat. Muscle (skeletal) A	6·1	0·86	2·14	0·035	
B	7·0	1·0	2·5	0·035	
C	7·0	0·92	2·3	0·033	
Blood (whole)	—	—	—	—	Completely absent.
Rabbit. Liver A (1) ...	20	19·2	48·0	0·24	Animal starved 16 hrs. to free liver from glycogen.
(2) ...	20	20·0	50·0	0·25	
B (1) ...	20	21·6	54·0	0·27	
(2) ...	20	22·4	56·0	0·28	
Rabbit. Muscle (skeletal)	15	2·4	6·0	0·04	
	15	2·4	6·0	0·04	
	14·8	2·6	6·5	0·044	
Kidney	8·0	5·6	14·0	0·17	Mean value.
Blood (whole)	20 cc.	—	—	—	Complete absence.
Human blood	20 ,,	—	—	—	Complete absence.
Egg (hen)	—	—	—	—	Absent.

The mean values obtained are summarised below:

		%	Range %
Skeletal muscle	Rat	0·034	
	Rabbit	0·04–0·045	
Liver	Rat	0·18	0·16–0·21
	Rabbit	0·24	0·22–0·35
Yeast (fresh)		0·18	0·15–0·22

The possibility that an equilibrium between the —SH and the —SS— forms of the dipeptide was disturbed during the excision of the tissue and subsequent manipulation (as in the case of lactic acid) was checked as far as possible by adopting a technique similar to that used by Hopkins and Fletcher [1907] in determining the resting minimum for lactic acid in muscle.

The tissue was removed as rapidly as possible after the death of the animal into a tared, ice-cold beaker, weighed and ground under ice-cold trichloroacetic acid in a mortar surrounded by ice. The whole procedure was carried out as far as possible at 0°. No significant differences were observed between the results obtained at 0° and those at ordinary temperatures.

Discussion of Results.

The assumption has been made that the whole of the soluble sulphydryl groups in the tissues are those of glutathione. At the present moment, there is no evidence that cysteïne exists as such free in normal tissues. Extracts made from large quantities of yeast have been concentrated but in no case was cysteine (or cystine) isolated.

There remains the possibility of course that other peptides of a nature similar to glutathione exist, but until such have been definitely isolated it seems justifiable and convenient to express the results as percentage of reduced glutathione.

The close approximation of the values for the organic sulphur and the sulphur calculated from the iodine titration (which estimates —SH groups only) suggests that by far the greater part of the glutathione exists in the tissues in the reduced form. This deduction has been confirmed experimentally as follows. Two equal portions of the same sample of yeast were taken. In one the reduced glutathione was estimated as described. The other was suspended in a phosphate buffer solution at p_H 7·6 in an evacuated tube and incubated at 37° for one hour. The estimation was then carried out in the same manner. No increase in the amount of reduced glutathione was found. The same was the case with liver and muscle.

If, however, oxidised glutathione was added to the sample, a considerable increase in the amount of reduced glutathione was found. The lack of any increase in the experiment was thus due not to the inability of the tissue to reduce the —S—S— to the —SH group, but to the absence of the —S—S— group.

This result is of interest since evidence is accumulating that the —SH group is the active group in the catalytic oxidation processes in which glutathione plays a part.

The liver is undoubtedly the richest organ, and it will be noticed that the organs of the rabbit have a somewhat larger content than the corresponding organs of the rat.

The figures obtained for skeletal muscle (both in the rat and the rabbit) have been exceedingly consistent; those for the liver were variable. The variations in the case of the liver may in part be due to differences in the amount of blood remaining in the organ after bleeding the animal.

In cases where bleeding was poor, and the organ distended with blood, the percentage was always rather lower than usual. The effect of loss of moisture is well shown in the case of yeast; consequently fresh samples were obtained for each determination.

The values obtained by this method of estimation are from 3–5 times greater than the amounts actually isolated.

When the possibilities of loss in the various stages of the separation are considered, the difference may be well accounted for. At one stage alone—precipitation with mercuric sulphate reagent—losses of the order of 40 % have been recorded with cystine [Hopkins, 1921] and a similar loss is probable in the case of glutathione.

Abderhalden and Wertheimer [1923] have estimated colorimetrically the substance reacting with nitroprusside in horse muscle. This, calculated as cysteine, was present to the extent of 100 mg./kg. As glutathione, this on recalculation gives 0·02 %.

The method used by these authors depends on the matching of the nitroprusside colour with a standard mixture of dye-stuffs (Bordeaux red and methylene blue). The nitroprusside colour fades with exceptional rapidity and although, as stated by Abderhalden and Wertheimer, the colour may be made to persist to some extent by the addition of potassium cyanide, the sources of error would appear to be considerable.

It has already been pointed out that no evidence exists for the presence of free cysteine in the tissues of normal animals; and only in cases of cystinuria has the existence of cystine in the tissues been shown.

SUMMARY.

(1) Glutathione in the tissues of normal animals is chiefly in the reduced form (—SH).

(2) The sulphur equivalent to the —SH groups present accounts for the greater part of the soluble "neutral" sulphur of tissue extracts.

(3) The glutathione content of certain tissues has been estimated and values are given.

My thanks are due to Sir F. G. Hopkins, F.R.S., for his encouragement during the progress of this work.

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XXVIII. GLUTATHIONE.

RELATION BETWEEN THE TISSUES AND THE OXIDISED DIPEPTIDE.

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(*Received January 21st, 1925.*)

In his original paper on glutathione Hopkins [1921] showed that the hydrogen-ion concentration of the medium had a considerable effect on the velocity of reduction of methylene blue by tissues. This was the case both with fresh tissues and with washed tissues artificially supplied with oxidised glutathione.

He noted that, whereas at reactions slightly on the alkaline side of neutrality the addition of the oxidised dipeptide markedly accelerated the reduction of the dye, alteration of the reaction to the acid side of the neutral point resulted in a retardation of the reduction on the addition of the dipeptide. The explanation was advanced that the —SH groups formed by the reduction of the disulphide form were unable to transfer their hydrogen at an acid reaction, and thus the oxidised glutathione was acting as a competitor with the methylene blue for the available labile hydrogen of the tissues.

In view of the later results of Dixon and Tunnicliffe [1923], who showed that even at p_H 5·0 reduced glutathione was able to reduce methylene blue, it appeared to be of value to continue the investigation of the effect of p_H on the systems described by Hopkins, and Hopkins and Dixon [1922].

EXPERIMENTAL.

Methylene blue technique.

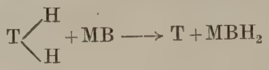
Details of a typical experiment are given below.

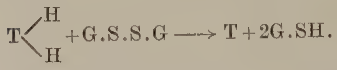
Sheep liver was minced and washed six times with distilled water. It was then ground under alcohol, dried and again washed with distilled water. To each of a series of tubes were added 0·5 g. of the tissue, 0·3 cc. of 1/5000 methylene blue solution, and the requisite quantities of oxidised glutathione and phosphate buffer solutions. In each case the total volume was kept the same. The tubes were then evacuated and placed in the water-bath at 37°. The times for decolorisation of the dye were noted and are tabulated below.

Oxidised glutathione mg.	Reduction time (minutes) at p_H 7·6	Reduction time (minutes) at p_H 6·2
0	145	245
2	120	210
4	90	180
8	40	122

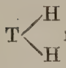
Similar results have been obtained with both heated and unheated preparations of the muscle of the rabbit, rat, dog and cat. In no case within the limits of p_H examined has there been observed a retarding effect on the addition of oxidised glutathione.

The reactions occurring in the system may be represented as follows:

(i) 
$$\mathrm{T}\begin{smallmatrix}\diagup \mathrm{H}\\ \diagdown \mathrm{H}\end{smallmatrix} + \mathrm{MB} \longrightarrow \mathrm{T} + \mathrm{MBH_2}$$

(ii) 
$$\mathrm{T}\begin{smallmatrix}\diagup \mathrm{H}\\ \diagdown \mathrm{H}\end{smallmatrix} + \mathrm{G.S.S.G} \longrightarrow \mathrm{T} + 2\mathrm{G.SH}.$$

(iii) $2\mathrm{G.SH} + \mathrm{MB} \longrightarrow \mathrm{G.S.S.G} + \mathrm{MBH_2}$

where  $\mathrm{T}\begin{smallmatrix}\diagup \mathrm{H}\\ \diagdown \mathrm{H}\end{smallmatrix}$ represents the hydrogen donator of the tissues.

MB and MBH_2 methylene blue and methylene white.
G.S.S.G and G.SH the oxidised and reduced forms of the dipeptide respectively.

The above result shows that the direct reaction (i) is slowed by increasing acidity. The combined reactions (ii) and (iii) proceed at a greater rate than the single direct reaction no matter whether the p_H be on the acid or alkaline side of the neutral point. As might be expected from the results of Dixon and Tunnicliffe [1923] the whole process (i), (ii) and (iii) proceeds more rapidly in alkaline than in acid solutions. Further analysis of the results is rendered somewhat difficult owing to the complicated nature of the system. The fact that the reaction (iii) is slowed by increasing acidity is suggestive that the effect of p_H on the whole system is due to its effect on reaction (iii).

The method throws no light on what is perhaps the most interesting of the reactions—that between the hydrogen donators of the tissue and the oxidised glutathione. An attempt has therefore been made to attack the problem directly.

The method used was as follows. A known weight of the tissue preparation was suspended in a solution of oxidised glutathione at the required p_H and the tube exhausted. After being kept for the necessary time at 37° the tube was opened and the reduced glutathione estimated as described in a previous paper [Tunnicliffe, 1925]. The end point can be determined either by the use of starch, or by using sodium nitroprusside as an external indicator in the usual manner. In the case of heated tissue preparations the end point with starch is fairly sharp, but in the case of the cold washed preparation it is preferable to use the external indicator. The results by the two methods agree fairly closely.

The results of a typical experiment showing the course of reduction of oxidised glutathione by a muscle preparation are given graphically in Fig. 1.

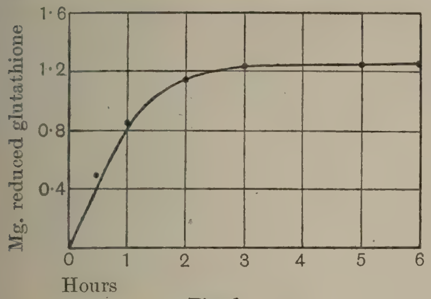


Fig. 1.

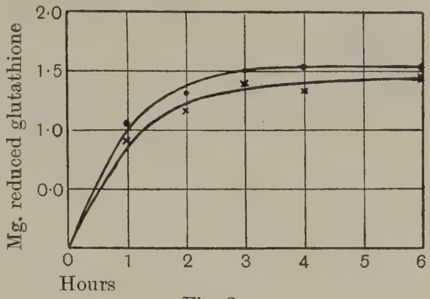


Fig. 2.

Fig. 1. Reduction of oxidised glutathione by muscle.
Thermostable preparation of cat muscle, 0·2 g.
Oxidised glutathione, 8 mg.
Phosphate buffer solution, p_H 7·6.

Fig. 2. Reduction of oxidised glutathione by muscle.
—×—×— Washed, heated.
—●—●— Washed, unheated.
Equal weights of washed unheated rabbit muscle were used for each determination in the two series. The muscle in the second (heated) series was plunged for 5 minutes into boiling water before use.
Phosphate buffer solution, p_H 7·6.

A series of tubes was used and estimations made on separate tubes at suitable intervals. It will be observed that the reduction occurs rapidly for the first three hours, and that after this time the rate of the reaction diminishes until after five hours the reaction is complete. The amount of reduced glutathione formed is considerably less than the amount of oxidised glutathione added. This point will be referred to again in a later section of the paper.

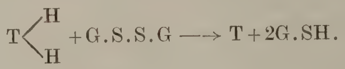
In view of the statements of Abderhalden and Wertheimer [1923] that frog muscle can be washed until it shows no power of reducing cystine, and that the reducing power can be restored by the addition of "Kochsaft," or by heating, these points were examined in some detail.

On exposure of various tissue preparations to the air it has frequently been observed that there was a perceptible diminution in the capacity of reducing the oxidised dipeptide; particularly when exposed in thin layers in the moist state. Hopkins and Dixon [1922] also noted this fact. For this reason the tissues have been washed anaerobically by shaking with water in a stoppered cylinder through which nitrogen had been bubbled.

Frog muscle was minced by a special machine to a much finer state of division than can be obtained by chopping with scissors. It was then washed anaerobically with boiled tap water until all traces of haemoglobin were removed, and finally shaken in nitrogen for 4 hours with distilled water and toluene. After drying with alcohol in the usual manner the preparation was found to reduce oxidised glutathione and cystine vigorously.

The effect of heating the tissue preparation for a short time at 100° on the course of the reduction was also examined. The results are shown in Fig. 2 (p. 201). The reaction proceeds somewhat more smoothly in the case of the heated preparation, but within the limits of experimental error the total reduction is the same in both cases. The alteration in permeability of the preparation due to the heating would in all probability account for the greater ease of reduction in the case of thermostable preparations.

As mentioned previously the amount of oxidised glutathione added to the tissue preparation was in excess of the reducing capacity of the tissue used. An experiment was therefore carried out using in each case the same weight of muscle preparation but gradually increasing the amount of oxidised glutathione added. The reaction was allowed to proceed in each case for 6 hours and the reduced glutathione then estimated. In Fig. 3 the abscissae represent the amounts of oxidised dipeptide added and the ordinates the amounts of reduced glutathione formed. The results show clearly that below a certain amount the oxidised glutathione is practically completely reduced; after this point further increase in the amount of the oxidised form leads to no further formation of reduced glutathione. This maximum amount of oxidised glutathione which a given weight of a tissue will reduce is constant within the limit of experimental error for any given preparation, and is to be regarded as a measure of that labile hydrogen of the tissue which is concerned in the reaction



$$T\begin{matrix}\diagup H\\ \diagdown H\end{matrix} + G.S.S.G \longrightarrow T + 2G.SH.$$

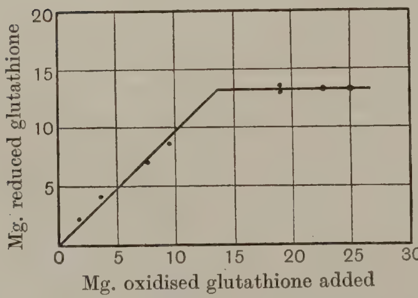


Fig. 3.

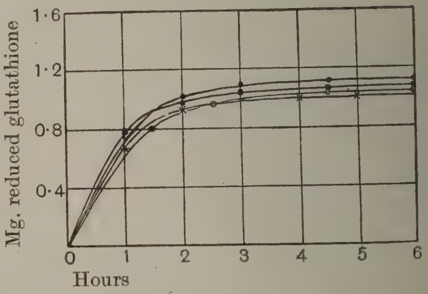


Fig. 4.

Fig. 3. Relation between amount of reduction and amount of oxidised glutathione present. For each determination 2 g. of a thermostable preparation of cat muscle was used, suspended in phosphate buffer solution p_H 7·6.

Fig. 4. Effect of p_H and nature of medium on course of reduction.
—●—Phosphate buffer solution p_H 7·6.
—■— ,, ,, ,, p_H 8·0.
—×— ,, ,, ,, p_H 6·0.
—⊖— Solution adjusted to p_H 7·6 with dilute Na_2CO_3 solution.

The effect of changes in the nature of the medium in which the tissue was suspended was next examined. The results shown in Fig. 4 may briefly be stated as follows. No significant difference in the rate or amount of reduction could be detected by altering the p_H or the salts of the medium. Thus there was no difference between the course of reduction in phosphate buffer solution at p_H 7·6, and that in a dilute sodium carbonate solution of approximately the same p_H.

The bearing of the facts above may now be considered in relation to the complete system (Tissue + Oxidised Glutathione + Hydrogen Acceptor). Where methylene blue is the hydrogen acceptor a clear conception of the process can be obtained.

The first point of interest is that the amount of reduced glutathione formed by a given weight of a tissue preparation in contact with excess of the oxidised dipeptide corresponds extremely well with the maximum amount of methylene blue which the same weight of the preparation will reduce. Thus 1 g. of a muscle preparation was found to reduce 6·5 mg. of the dipeptide. The same amount of tissue was found by the method described by Hopkins and Dixon [1922] to reduce between 4·8 and 4·9 cc. of 1/1000 solution of methylene blue. Calculating from the equation $2G.SH + MB = G.S.S.G + MBH_2$ this is equivalent to 6·4 mg. of reduced glutathione.

The result of alterations of hydrogen-ion concentration on the system is also clearly explained. At p_H 7·6 and 6·0, for example, the rate of the reaction $T\langle^{H}_{H} + G.S.S.G$, as shown above, will be the same in both cases. The rate of the final reaction $2G.SH + MB$ will however be considerably less at the lower p_H and consequently the time for reduction of the methylene blue will be greater in the second of the two cases.

When oxygen is the hydrogen acceptor the bearing of the results described is more interesting and at the same time more complex. Calculating, as in the case of methylene blue, the volume of oxygen equivalent to the reduced glutathione formed by 1 g. of a thermostable muscle preparation, the oxygen uptake is found to be of the order of 140–150 mm.^{3}. This figure is of course unaffected by the nature of the medium in which the tissue is suspended, for, as has been pointed out, the amount of reduced glutathione formed is independent of changes in the medium.

The oxygen uptake actually measured in the Barcroft apparatus is in phosphate buffer solution 400 mm.^{3} per g. of tissue preparation. In Ringer solution or in the absence of phosphate ions the measured uptake is increased to four times this amount, *i.e.* 1600 mm.^{3} per g. approximately.

There seems no doubt therefore that in addition to the oxidation of those substances which anaerobically are solely connected with the reduction of oxidised glutathione, there must also occur an aerobic oxidation of some other substance associated with the thermostable tissue residue. This additional oxidation occurs in the presence of phosphate ions but to a very much larger extent in their absence. Meyerhof [1923] has recently shown that the oxidation of an unsaturated fatty acid, linolenic acid, by oxygen, is greatly accelerated by the presence of a sulphydryl group which is also undergoing oxidation at the same time. The oxidation of the linolenic acid in presence of a sulphydryl group is greatly diminished by phosphate ions. Meyerhof used as a sulphydryl compound thioglycollic acid. He also showed that the alcohol-ether extract of thermostable muscle preparations contained an unsaturated acid associated with the lecithin fraction. Through the kindness of Professor Hopkins I am able to state that the oxidation of unsaturated fats and fatty acids, in particular linolenic acid, by oxygen is greatly increased when the reduced form of glutathione is simultaneously undergoing oxidation. Phosphate ions also inhibit this oxidation of the double bonds of linolenic acid in the presence of reduced glutathione.

There seems therefore to be no doubt that the oxidation of the unsaturated fatty acid is the cause of the discrepancy between the oxygen uptake as measured in the Barcroft apparatus, and the uptake calculated from the amount of reduced glutathione which the preparation will form anaerobically.

Meyerhof however appears to regard the unsaturated acid as being the substance responsible for the reduction of the disulphide (dithiodiglycollic acid in his experiments) to the sulphydryl form according to the equation

$$\text{—CH} = \text{CH—} + \begin{matrix}\text{HOH}\\ \\ \text{HOH}\end{matrix} + \text{T.SS.T} \longrightarrow \text{—CHOH—CHOH—} + 2\text{T.SH}.$$

This view is at variance with the results which have been described above, and the following experiments were therefore carried out to determine whether there was such a possibility in the case of glutathione. The substances shown in the table below were kept in evacuated tubes in the water-bath at 37° for 20 hours.

Tube	Linolenic acid mg.	Oxidised glutathione mg.	Medium	p_H	Methylene blue 1/5000 cc.
1	20	10	Borate	6·5	0·0
2	20	10	,,	6·5	0·3
3	20	10	,,	7·8	0·0
4	20	10	,,	7·8	0·3
5	20	10	,,	8·4	0·0
6	20	10	,,	8·4	0·3

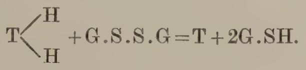
Tubes 1, 3 and 5 were opened and the nitroprusside test was found to be negative in each case. There was therefore no reduction of the disulphide form to the sulphydryl form. This was confirmed by the fact that the methylene blue was not reduced in tubes 2, 4 and 6, although there was a strong adsorption of the dye on the surface of the oil droplets which separated out.

It is thus clear that linolenic acid is unable to reduce oxidised glutathione; if such reduction did take place the fact (communicated to me by Professor Hopkins) that the disulphide form causes no increase in the oxidation of the linolenic acid by atmospheric oxygen, would be meaningless.

This has also been confirmed as follows: thermostable residues were extracted for 6 hours in a Soxhlet apparatus with alcohol, ether and acetone in the order described. The total extraction lasted 18 hours and should have removed thoroughly any fatty acid which was present. Despite this violent treatment the tissue residue still possessed over 50 % of its original capacity for reducing the oxidised dipeptide.

What diminution was observed may well have been due to oxidation of the hydrogen donators of the tissue during the extraction, since ether when boiled tends to form an ether peroxide.

The picture of the aerobic oxidation process suggested previously is thus supported. The linolenic acid is oxidised concurrently with the oxidation of the sulphydryl groups of the glutathione which are established by the reduction of the disulphide form by the hydrogen donators of the tissues. So long as the supply of the donators remains unexhausted there will be a cyclic process involving the alternate reactions

(i) 

$$\mathrm{T}\begin{matrix}\diagup \mathrm{H} \\ \diagdown \mathrm{H}\end{matrix} + \mathrm{G.S.S.G} = \mathrm{T} + 2\mathrm{G.SH}.$$

(ii) $4\mathrm{G.SH} + \mathrm{O_2} = 2\mathrm{G.S.S.G} + 2\mathrm{H_2O}.$

Concurrently with reaction (ii) will occur the oxidation of the double bonds of the unsaturated acid. When the hydrogen donators are exhausted reaction (i) ceases, the sulphydryl groups are oxidised, and the system ceases to take up oxygen. With the disappearance of the sulphydryl groups the oxidation of the double bonds of the unsaturated fatty acid ceases.

The effect of alteration of the hydrogen-ion concentration on the aerobic process remains to be considered. At p_{H} 7·6 the oxidation of reduced glutathione proceeds very rapidly compared with the rate at which the tissue can reduce the oxidised dipeptide. A very low concentration of the sulphydryl form would therefore be expected. This is in agreement with fact, for under the conditions described the nitroprusside reaction is exceedingly faint. The amount of reduced glutathione present was insufficient for estimation by the method described. At p_{H} 6·0 the rate of oxidation of the reduced glutathione is much slower than at p_{H} 7·6. As the rate at which the oxidised dipeptide is reduced is unaltered by alteration of p_{H} a higher concentration of the —SH form is therefore to be expected at the lower p_{H}, other conditions being the same in the two cases. This is also confirmed experimentally, both by estimation and by means of the intensity of the nitroprusside reaction, particularly in the earlier stages of the aeration of the system.

In cases where the dipeptide is originally present in the reduced form, the conditions will obviously be different from the cases in which it was added in the oxidised form. At a p_{H} of 7·6 the oxidation of the —SH groups already present will proceed with considerable rapidity and will be practically complete in one hour. There will thus be an initial rapid uptake of oxygen, after which the conditions will approximate closely to those in the system commencing with the glutathione in the —S—S— form. The final total uptake of oxygen will be greater in the case where the glutathione was originally in the —SH form, by an amount of oxygen equivalent to this quantity of —SH groups.

At a more acid reaction, *e.g.* p_H 6·0, the oxidation of the —SH groups already present will be very much slower than at p_H 7·6 and there will thus be a higher concentration of the —SH form at the lower p_H.

The exact effect of the varying concentrations of —SH groups on the velocity of oxygen uptake by the system is difficult to predict; the fact that the disulphide form as shown by Dixon and Tunnicliffe accelerates the oxidation of the reduced form of the dipeptide [1923], and the close relation between the oxidation of the unsaturated fatty acids and that of the —SH groups introduce factors which render such an attempt at present inadvisable. The ultimate solution will rest on a further knowledge of the kinetics of the reaction

$$4G.SH + O_2 = 2G.S.S.G + 2H_2O$$

both in the absence and presence of unsaturated fatty acid linkages.

It is hoped to continue the investigation from this point of view.

SUMMARY.

(1) A study has been made of the reaction between the thermostable tissue residue and oxidised glutathione.

(2) The bearing of the results obtained is discussed with reference to the reduction of methylene blue, and to the uptake of oxygen by tissue residues in the presence of oxidised glutathione.

(3) Linolenic acid was found not to be the substance in the thermostable residue responsible for the reduction of oxidised glutathione.

I am greatly indebted to Sir F. G. Hopkins, F.R.S., for allowing me to use certain of his results, and for his constant interest during the course of the work.

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XXIX. GLUTATHIONE.

SYNTHESIS.

By CORBET PAGE STEWART
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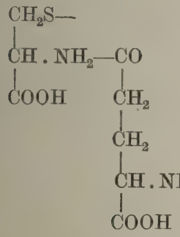
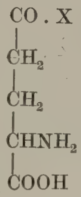
In a previous paper [1923] the authors, with Mr J. H. Quastel, put forward the view that glutathione possesses the constitution represented by Formula I.



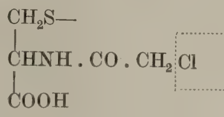
$$\begin{array}{ll} CH_2S- & \\ | & \\ CH.NH_2- & CO \\ | & | \\ COOH & CH_2 \\ & | \\ & CH_2 \\ & | \\ & CH.NH_2 \\ & | \\ & COOH \end{array}$$

I.



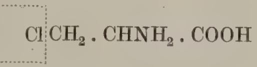
$$\begin{array}{l} CO.X \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ COOH \end{array}$$

II.



$$\begin{array}{l} CH_2S- \\ | \\ CHNH.CO.CH_2Cl \\ | \\ COOH \end{array}$$

III.



$$Cl\,CH_2.CHNH_2.COOH$$

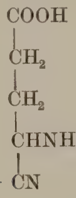
IV.

The evidence on which they based that view was derived from a study of the breakdown of the glutathione molecule in various directions. The fact that condensation of glutathione with 2 . 3 . 4-trinitrotoluene (which condenses with primary amine groups) and subsequent hydrolysis of the product yielded free cystine showed that in glutathione the free amino group was that of the glutaminic portion. Confirmation of this was given by treatment with nitrous acid followed by hydrolysis, when α-hydroxyglutaric acid was isolated. Oxidation by hydrogen peroxide gave a compound from which succinic acid was obtained after hydrolysis. This, by analogy with the work of Dakin [1905] on the oxidation of amino acids, was held to indicate that that carboxyl group of glutaminic acid remote from the amino group was involved in the peptide linkage. It was pointed out, however, that the constitution of glutathione could only be regarded as definitely established when a substance of the proposed formula had been synthesised and shown to be identical with the natural product.

The problem presented in the synthesis—that of linking the amino group of cystine with that carboxyl group of glutaminic acid which is remote from the amino group—resolved itself into the preparation of the appropriate mono-acid halide of glutaminic acid (II) or of some substance which can subsequently be converted to glutaminic acid.

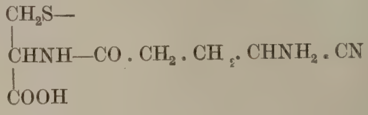
Alternatively, the following synthesis, which, though indirect, would definitely establish the constitution, was considered. Cystine readily condenses with chloroacetyl chloride [Fischer and Suzuki, 1904] giving dichloroacetyl-cystine (III). By elimination of chlorine from this and β-chloroalanine (IV) glutathione should result. This method was abandoned, however, not only because of its tedious nature—it involves the preparation of serine—but also because it was found that the condensation of even simple chloro compounds in the presence of "molecular" silver, sodium, etc., was extremely unsatisfactory.

The preparation of the compound V by Strecker's reaction on the semi-aldehyde of succinic acid presents itself as a solution until it is remembered that after halogenation and coupling of the acid halide to obtain the compound VI, prolonged hydrolysis would be necessary to convert the nitrile group to the required carboxyl group. Such hydrolysis would certainly disrupt the peptide linkage and thus defeat the end in view.



$$\begin{array}{l} COOH \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ CHNH_2 \\ | \\ CN \end{array}$$

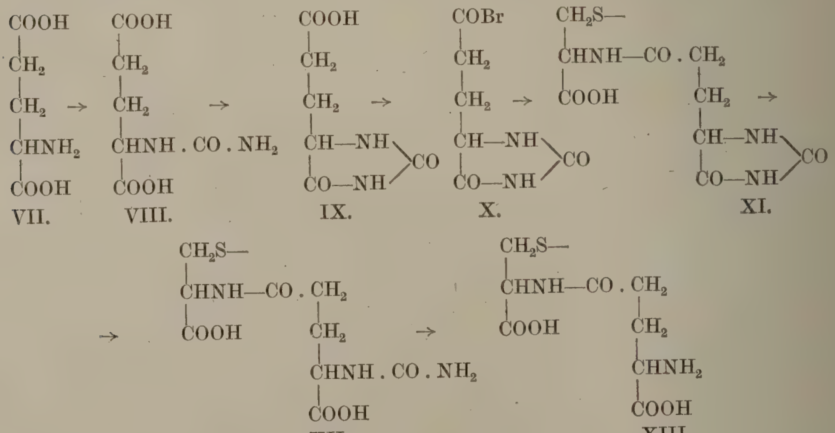
V.



$$\begin{array}{l} CH_2S— \\ | \\ CHNH—CO\,.\,CH_2\,.\,CH_2\,.\,CHNH_2\,.\,CN \\ | \\ COOH \end{array}$$

VI.

The method finally adopted is shown in the scheme below.



Glutaminic acid (VII) was converted to the hydantoinpropionic acid (IX) by the method of Dakin [1919]. This, on treatment with phosphorus tribromide is converted to the acid bromide (X) which is then coupled with cystine dimethyl ester hydrochloride [Fischer and Suzuki, 1905] in the ordinary way. The opening of the hydantoin ring presents a problem similar to that encountered by Dakin in his synthesis of β-hydroxyglutaminic acid. Boiling with barium hydroxide, he found, brought about complete decomposition of the bulk of his substance (hydantoin-β-bromopropionic acid). Hydantoin-acrylic acid, on prolonged treatment with the same reagent, gave off ammonia and carbon dioxide with formation of the amino acid, but considerable losses were again experienced. His alternative method of heating in a sealed tube with fuming hydrochloric acid at 130° is obviously inadmissible in the present case owing to the presence of a peptide linkage. Boiling for a short time with calcium hydroxide, however, appeared to open the ring with, probably, the formation of a uramino acid (XII), and with the destruction of only a small amount of cystine. Rohde [1918] has shown that uramino acids, treated with nitrous acid, lose half their nitrogen. She did not, however, identify the products of the reaction. Dakin (private communication) has found that from β-*p*-hydroxyphenyl-α-uraminopropionic acid, under such treatment, tyrosine can be obtained in considerable yield. It is reasonable to suppose that glutaminyl-cystine-uramino acid (XII) would behave in the same way, and accordingly the solution of the substance obtained by the action of calcium hydroxide on di-hydantoinpropionyl-cystine (XI) is treated with nitrous acid in slight excess of the amount required for the removal of one nitrogen atom. The isolation of the dipeptide (XIII) obtained in this reaction is accomplished through the mercuric sulphate compound.

Whilst the substance so obtained agrees with the natural substance in chemical properties and in certain physical properties, it has not the same optical activity. This is most probably due to racemisation during the formation and subsequent opening of the hydantoin ring [Dakin, 1910]. The agreement in all other respects, however, is so close that it is difficult to imagine any difference between the synthetic and natural substances save that of configuration. The differences between the three *possible* constitutions of glutathione are so marked, that did glutathione not possess the constitution proposed, and, consequently, not that of the synthetic product, the two substances must inevitably differ more widely. Especially is this true of the melting point, even though such a criterion is of lessened value when one is dealing with amorphous substances.

Nevertheless, it seemed desirable, since resolution of the racemic peptide appeared a well nigh hopeless task, to attempt a synthesis, which, while yet affording confirmatory evidence of the constitution, should give a substance completely identical with natural glutathione. This object was successfully attained by a direct coupling of cystine and glutaminic acid.

Glutaminic acid was found to react with phosphorus tribromide. The ease with which the product of this reaction was hydrolysed, and its ability to condense with aniline in slightly alkaline solution, showed it to be an acid bromide. Analysis of the anilide gave figures in close agreement with those required for the hitherto unknown glutaminyl mono-anilide. The reaction of phosphorus tribromide and glutaminic acid had therefore yielded a *mono*-acid bromide. The two-fold object of the synthesis—to confirm the constitution and to obtain a product identical with natural glutathione—demanded that before proceeding further, the constitution of this acid bromide should be definitely established. For this purpose glutaminyl mono-anilide was prepared in sufficient quantity, and use was made of the oxidation method employed in determining the constitution of glutathione itself. It was there argued [Quastel, Stewart and Tunnicliffe, 1923] that the two compounds: $R.NH.CO.CH_2.CH_2.CHNH_2.COOH$ and $R.NH.CO.CHNH_2.CH_2.CH_2.COOH$ should behave differently on oxidation with hydrogen peroxide. Dakin [1905] has shown that α-amino acids, treated in this way, eventually yield fatty acids containing one carbon atom less, glutaminic acid for example, being converted to succinic acid. The first, therefore, should yield $R.NH.CO.CH_2.CH_2.COOH$ which, on hydrolysis, should be broken up to $R.NH_2$ and succinic acid. The second should yield $R.NH.CO.CO.CH_2.CH_2.COOH$ which further oxidation should split up, possibly to succinic acid. At any rate, the complete breakdown should, in this case, take place before hydrolysis. In the particular case of glutaminyl mono-anilide, the problem is simplified by the fact that succinyl mono-anilide is known (see Beilstein's *Handbuch III*, II, 413) so that hydrolysis after oxidation is unnecessary. In actual fact, oxidation of glutaminyl mono-anilide yielded a substance extractable by ether, which was identified by properties, melting point, and analysis, with succinyl mono-anilide. The acid bromide from which it was derived was thus represented by the formula:

$$BrOC.CH_2.CH_2.CHNH_2.COOH.$$

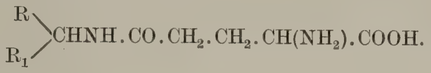
That is, it had the bromine atom attached to the carboxyl group remote from the amino group, and was therefore the one required for the synthesis of a compound having the constitution proposed for glutathione.

Condensation of the acid bromide with cystine yielded a dipeptide which had all the properties of natural glutathione, and which, on oxidation with hydrogen peroxide behaved in exactly the same way, yielding succinic acid only after hydrolysis of the oxidation product.

The comparison of the physiological properties of the natural and synthetic dipeptides has not yet been fully worked out. It has been observed, however, that like natural glutathione, both the synthetic products restore to a thermostable preparation of dog muscle the power of reducing methylene blue. Further, when either of them is added in "oxidised" (disulphide) form to such a preparation, and is incubated *in vacuo* at 37° for a few minutes, the —S—S— grouping is reduced to —SH.

Both the methods of synthesis involve the coupling of an acid bromide with cystine dimethyl ester. In both of these reactions considerable losses were experienced, so that the final yields of dipeptide were small. Little better, however, is to be expected in such a case where, besides the main reaction, an acid bromide and an ester are undergoing simultaneous hydrolysis in a slightly alkaline medium.

The methods described in this paper, are, of course, generally applicable to the synthesis of dipeptides of the type:



$$\begin{matrix} R \\ R_1 \end{matrix}\rangle CHNH.CO.CH_2.CH_2.CH(NH_2).COOH.$$

Experimental.

Synthesis I.

Preparation of the Acid Bromide of Hydantoinpropionic Acid.

The dry, finely powdered hydantoinpropionic acid was covered with excess of phosphorus tribromide, and the mixture was heated on the water-bath for two to three hours in a wide-mouthed flask under a reflux condenser. The yellowish coloured mass was freed from as much as possible of the excess of phosphorus tribromide by decantation, and the remainder was removed by repeated trituration with dry chloroform. It was then transferred to a vacuum desiccator containing sulphuric acid and paraffin wax. The bromine content of the product was estimated by hydrolysis for 20 minutes with excess of a twice normal solution of potassium hydroxide, acidification with nitric acid, and precipitation of the silver bromide with silver nitrate.

0·512 g. of the product gave 0·369 g. of silver bromide. Hence 90 % of the hydantoinpropionic acid had been converted to the acid bromide. No further purification of the crude product was attempted.

Preparation of Di-hydantoinpropionyl-cystine.

5 g. of cystine dimethyl ester hydrochloride, prepared according to the method of Fischer and Suzuki [1905], were dissolved in 50 cc. of ice-cold water giving a clear solution. After further cooling in a freezing mixture (as short as possible since the ester begins to be hydrolysed at once) solid sodium bicarbonate was added until the solution was just alkaline to methyl red. While still in the freezing mixture, the solution was vigorously stirred by a mechanical device, and alternate small portions of the hydantoinpropionyl bromide and solid sodium bicarbonate were added, the solution being kept just alkaline to methyl red, excess being avoided, and 30 % excess over calculated quantity of the acid bromide was added—in the case described, 11 g. At the completion of the reaction the p_H was adjusted to the neutral point (to methyl red). Standing overnight completed the precipitation of unchanged cystine, which was then filtered off. The filtrate contained di-hydantoinpropionyl-cystine along with hydantoinpropionic acid, traces of cystine, and sodium salts. It was acidified with sulphuric acid, and treated

with a little dilute solution of sodium nitrite. Any uncombined cystine was thus destroyed, whereas the di-hydantoinpropionyl-cystine was unattacked owing to the absence of any free amino group from the molecule. The solution was then precipitated with the Hopkins-Cole mercuric sulphate reagent, and, after standing overnight, the heavy white precipitate was filtered at the pump and washed thoroughly with distilled water. Since hydantoinpropionic acid is not precipitated by mercuric sulphate, this precipitate consisted solely of the mercuric sulphate compound of di-hydantoinpropionyl-cystine. It was suspended in water and decomposed by hydrogen sulphide in the usual way. The filtrate was freed from hydrogen sulphide by aeration, and then made alkaline with barium hydroxide. The aeration was then continued until the vivid nitroprusside reaction had disappeared, barium was removed quantitatively with sulphuric acid, and the filtrate was concentrated *in vacuo* to a syrup. Considerable excess of a mixture of one part of absolute alcohol to two parts of dry ether was then added and the sticky product which separated was allowed to stand under the mixture until it became friable. It was filtered off rapidly and dried in a vacuum desiccator containing sulphuric acid, phosphorus pentoxide, and paraffin wax.

The product so obtained, which had a slight yellow colour, was exceedingly soluble in water giving an acid solution. It was insoluble in ether and in acetone. It was precipitated from aqueous solution by mercuric sulphate. The aqueous solution gave no nitroprusside reaction, but after reduction with magnesium and dilute hydrochloric acid an intense reaction was obtained. Heated in a capillary tube, it softened at 140° and melted at 174° (uncorr.).

Nitrogen by Micro-Kjeldahl. Found: $N = 15{\cdot}15\ \%$. Calculated for $C_{18}H_{24}O_{10}N_6S_2$: $N = 15{\cdot}32\ \%$.

Amino Nitrogen by the method of Van Slyke. Traces only of nitrogen were evolved. After hydrolysis, however, a qualitative test showed the presence of free primary amino groups.

Sulphur was determined by fusion with sodium peroxide. Found: $S = 11{\cdot}32\ \%$. Calculated: $S = 11{\cdot}68\ \%$.

1 g. of cystine was recovered from the precipitate which separated during the coupling of the cystine dimethyl ester with the acid bromide. Even allowing for this recovery, however, the yield was poor, as seems to be usual in reactions of this type. Part of the loss may no doubt be referred to the use of mercuric sulphate as a precipitant for cystine and its derivatives [Hopkins, 1921]. From 5 g. of cystine dimethyl ester hydrochloride 0·8 g. of di-hydantoinpropionyl-cystine was isolated.

Opening of the Hydantoin Ring.

The di-hydantoinpropionyl-cystine was dissolved in water and a moderate excess of finely-powdered calcium hydroxide was added. The mixture was boiled gently under a reflux condenser for three-quarters of an hour. At the end of that period the undissolved calcium hydroxide was filtered off and the filtrate was acidified with dilute sulphuric acid so that the resulting concentration of acid was approximately 1·5 %. On acidification, a certain amount of hydrogen sulphide was evolved showing that a partial decomposition of the cystine portion of the molecule had occurred. This decomposition, however, was less than with sodium or barium hydroxide, the use of which, moreover, would probably result in a greater disruption of the peptide linkage. After standing, the precipitated calcium sulphate was filtered off. No attempt was made to isolate the uramino acid from this solution; it was, however, observed to be precipitated by the mercuric sulphate reagent.

Conversion of the Uramino Acid to the Amino Acid.

Rohde [1918] observed that a uramino acid, treated with nitrous acid, loses half its amino nitrogen. Dr H. D. Dakin, in a private communication, states that by the action of nitrous acid on β-p-hydroxyphenyl-α-uraminopropionic acid, he has obtained tyrosine. Presumably, an imino acid is first formed and subsequently loses carbon dioxide.

The solution obtained in the previous stage was therefore treated with a dilute solution of potassium nitrite in slight excess of the quantity required for the conversion of the uramino to the amino acid. The solution was allowed to stand several hours, and the dipeptide was then precipitated by mercuric sulphate reagent. The further procedure was as described in the isolation of di-hydantoinpropionyl-cystine. Usually this sufficed, but occasionally it was found advisable to purify the substance further by precipitation of the copper compound of its "reduced" (sulphydryl) form [Hopkins, 1921].

Properties of the Dipeptide.

The dipeptide as thus prepared was a white non-hygroscopic amorphous powder. Like natural glutathione, it was extremely soluble in water, but insoluble in alcohol, ether, and the usual organic solvents. Its aqueous solution was acid to litmus. In neutral solution it gave a violet coloration with triketohydrindene. It gave no coloration with sodium nitroprusside in ammoniacal solution except after reduction with magnesium and hydrochloric acid. On hydrolysis with sulphuric acid, cystine was isolated; with hydrochloric acid, glutaminic acid hydrochloride was isolated.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: 5·62 %.

It will be recalled that in the case of natural glutathione, Hopkins [1921] obtained a similar high result (6·8 %).

Amino Nitrogen after hydrolysis. After hydrolysis of the substance with 20 % sulphuric acid for six hours, the amino nitrogen was doubled, and, allowing for the somewhat high value to be expected in the presence of cystine, was equal to the total nitrogen. Found: 12·2 %. Calculated: 11·24 %.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·26 %. Calculated: N = 11·24 %.

Heated in a capillary tube, the substance softened from 165–170° and melted at 187°. A sample of natural glutathione, heated side by side with the synthetic substance, behaved in exactly the same way. Not too much reliance, perhaps, should be placed on the melting point of an amorphous substance, but not only did the two substances melt at precisely the same temperature, but the melt of the synthetic product behaved in the manner characteristic of glutathione, in that there was an evolution of gas, and the liquid ran up the sides of the tube. Further, the melting point was not lowered by mixing the natural and synthetic products.

Optical Activity.

There is thus good agreement between the properties of natural glutathione and of the synthetic dipeptide—and it may be noted that no dissimilarities were observed. The best criterion of complete identity is, of course, the optical activity. From the mode of preparation of the synthetic substance, involving as it does, the closing and opening of a ring by somewhat drastic means, it is obvious that a partial racemisation, at any rate, is to be expected. However, the specific rotation of glutathione being still unknown, it was thought of some interest, at this point, to measure the rotation both of the natural and the synthetic product.

The Specific Rotation of "Oxidised" Glutathione.

I. *In aqueous solution.* 15 cc. of solution in distilled water contained 0·5194 g. of "oxidised" glutathione. The rotation, measured in a 2·0 dm. tube, using the mercury green line, was (mean of six concordant observations) $\alpha = -6{\cdot}82°$. The above solution was diluted to twice its volume with distilled water. Mean of six concordant observations: $\alpha = -3{\cdot}40°$, whence

$$[\alpha]^{15°}_{\mathrm{Hg.\,j}} = -98{\cdot}3°.$$

II. *In acid solution.* 15 cc. of solution in 10 % hydrochloric acid contained 0·2597 g. of "oxidised" glutathione. Mean of six concordant observations: $\alpha = -3{\cdot}09°$, whence

$$[\alpha]^{15°}_{\mathrm{Hg.\,j}} = -89{\cdot}2°.$$

The Specific Rotation of the Synthetic Dipeptide.

10 cc. of solution in distilled water contained 0·545 g. of the synthetic dipeptide. The rotation, measured in a 1·0 dm. tube using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -1{\cdot}67°$, whence

$$[\alpha]^{15°}_{\mathrm{Hg.\,j}} = -30{\cdot}6°.$$

SYNTHESIS II.

Preparation of the acid bromide of glutaminic acid. 10 g. of free glutaminic acid were suspended in 50 cc. of dry toluene. To this suspension were added 3·0 cc. of phosphorus tribromide, *i.e.* a 50 % excess over the quantity theoretically required to introduce a bromine atom into one carboxyl group. The mixture was boiled gently under a reflux condenser for three to four hours. After cooling it was rapidly filtered at the pump, and the light yellow solid was repeatedly triturated with dry chloroform. It was dried over sulphuric acid and paraffin wax (*in vacuo*). The bromine content of the crude product was estimated in the manner described for the acid bromide of hydantoinpropionic acid. In a typical case, 0·4806 g. gave 0·123 g. of silver bromide, corresponding to a 30 % yield of a mono-acid bromide.

Condensation of the acid bromide with aniline. 4·0 cc. of aniline were suspended in 40 cc. of water and coupled with the acid bromide in the manner previously described for the condensation of hydantoinpropionyl bromide and cystine dimethyl ester (p. 211), except that the solution was not cooled below room temperature. 10 g. of the crude acid bromide were used. After removal of the excess aniline by repeated extraction with ether, the reaction mixture was acidified with hydrochloric acid, and evaporated to dryness *in vacuo*. The residue was extracted several times with absolute alcohol. The combined extracts were decolorised by boiling with a little animal charcoal, and were then evaporated on the water-bath. The crystalline residue was recrystallised from absolute alcohol.

The colourless spear-head crystals were readily soluble in water and alcohol, but insoluble in ether and acetone.

Nitrogen by Micro-Kjeldahl. Found: N = 10·4 %. Calculated for $C_6H_5NH.C_5H_8O_3N.HCl$: N = 10·83 %.

Chlorine by precipitation with silver nitrate. Found: Cl = 13·51 %. Calculated: Cl = 13·73 %.

Calculated for the di-anilide, $C_6H_5NH.C_5H_7O_2N.C_6H_5NH.HCl$:

$$N = 12{\cdot}59\ \%;\quad Cl = 10{\cdot}64\ \%.$$

Oxidation of glutaminyl mono-anilide. A solution in distilled water of the glutaminyl mono-anilide was made faintly alkaline with ammonia, a trace of ferric chloride was added, and the liquid was warmed on the water-bath with excess of hydrogen peroxide. As the hydrogen peroxide was used up, more was added until the reaction had been in progress for one-and-a-quarter hours. At the end of this time it was no longer possible to obtain a coloration with triketohydrindene. The solution was then acidified with hydrochloric acid, and thoroughly extracted with ether, the combined ethereal extracts being dried over sodium sulphate, and evaporated to dryness. There remained a mass of long, fine, colourless needles. These were dissolved in hot water, from which they separated on cooling, a qualitative test on the crude product having shown the presence of nitrogen. As, after recrystallisation, the test for nitrogen was still strongly positive, an estimation by micro-Kjeldahl was carried out. Found: N = 7·34 %. Calculated for $C_6H_5NH.C_4H_5O_3$: N = 7·25 %. The crystals melted sharply at 144–145°. In Beilstein's *Handbuch*, 3rd edition, **2**, p. 413, the melting point of succinyl mono-anilide is given as

148·5°. Later determinations, however, are rather lower than this for it is given (Ergänz. **2**, p. 210) as 144·5–145·5°. Melting point and nitrogen determination, then, agree in identifying the oxidation product as succinyl monoanilide. There is similar agreement in the reactions. The substance is sparingly soluble in cold water, but readily so in hot water, alcohol or ether. Its aqueous solution is acid to litmus. The addition of silver nitrate to its aqueous solution precipitates the crystalline silver salt. The barium salt is soluble, and cannot be obtained by adding barium salts (or hydroxide) to a solution of the anilide. The calcium salt, on the other hand, is sparingly soluble, and separates in small needles when calcium chloride solution is added to a neutral solution.

Condensation of glutaminyl mono-bromide with cystine dimethyl ester. The coupling was carried out exactly as described in the previous synthesis, care being taken to keep the temperature as low as possible during the reaction. As before, a variable amount of cystine was recovered from the reaction mixture. In one case, from 6 g. of cystine dimethyl ester, 3 g. of cystine were recovered. The dipeptide was isolated through its mercuric sulphate compound as previously described.

Identification of the dipeptide with natural glutathione. The substance thus prepared gave all the qualitative chemical reactions described in Hopkins' original paper [1921] and in this paper for the racemic dipeptide (p. 213). The melting point was identical with that of the natural product, and was not lowered by admixture.

Total Nitrogen by Micro-Kjeldahl. Found: N = 11·35 %. Calculated for $C_{16}H_{26}O_{10}N_4S_2$: N = 11·24 %.

Amino Nitrogen by the method of Van Slyke. Found: 6·4 %. Calculated: 5·62 %. As before, the value obtained for the amino nitrogen is high as is characteristic of the natural product and of cystine compounds in general.

Amino Nitrogen after hydrolysis. The substance was hydrolysed by boiling under reflux condenser for six hours with 20 % sulphuric acid. Found: amino N = 13·26 %. Calculated: amino N = 11·24 %. Found for natural glutathione [Hopkins, 1921]: amino N = 13·3 %.

Sulphur by the method of fusion with sodium peroxide. Found: S = 12·3 %. Calculated: S = 12·80 %.

Specific Rotation. 10 cc. of a solution in distilled water contained 0·5435 g. The rotation, measured in a 1·0 dm. tube, using the mercury green line, gave (mean of twelve concordant observations): $\alpha = -5{\cdot}295°$, whence

$$[\alpha]^{15°}_{\mathrm{Hg,\,j}} = -97{\cdot}4°.$$

SUMMARY.

I. Glutathione has been synthesised by two methods.

(*a*) Glutaminic acid—hydantoinpropionic acid—hydantoinpropionyl bromide—di-hydantoinpropionyl-cystine—di-uraminoglutaryl-cystine—di-glutaminyl-cystine (glutathione).

(*b*) Glutaminic acid—glutaminyl monobromide—di-glutaminyl-cystine.

II. The product obtained by the first of these methods differed from the natural substance only in its optical activity.

III. The second method yielded a substance completely identical with natural glutathione.

IV. The first method of synthesis substantiates the constitution of glutathione previously advanced by the authors.

V. It is shown that in the acid bromide of glutaminic acid used in the second synthesis, the bromine atom has entered the carboxyl group remote from the amino group. This synthesis, therefore, is also considered to confirm the constitution already deduced.

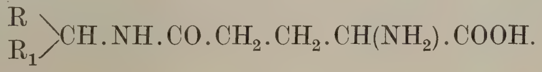
IV. The specific rotation of glutathione has been measured.
In aqueous solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -\,98{\cdot}3°.$$

In 10 % hydrochloric acid solution

$$[\alpha]^{15°}_{\text{Hg. j}} = -\,89{\cdot}2°.$$

VII. The methods described are generally applicable to the synthesis of dipeptides of the type:



$$\begin{matrix}\mathrm{R}\\ \mathrm{R_1}\end{matrix}\!\!>\mathrm{CH.NH.CO.CH_2.CH_2.CH(NH_2).COOH}.$$

The authors have gratefully to acknowledge valuable advice from Dr H. D. Dakin. They also wish to thank Sir F. G. Hopkins, F.R.S., for his constant encouragement.

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XXX. THE EFFECT OF HALOGEN SALTS ON SALIVARY DIGESTION.

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(*Received January 30th, 1925.*)

The experiments described in this paper were suggested by a mishap in an examination. The problem set the students was to find the effect of a given solution on the rate of salivary digestion of starch. The solution was to have been normal potassium fluoride. From experiments previously carried out in the laboratory this was found to delay the appearance of the achromic point considerably, so that instead of its showing in 3–8 minutes it took over 1 hour. For the examination it was necessary to buy a fresh supply of the fluoride, and for reasons of economy it was decided to obtain the sodium salt.

A normal solution of this sodium fluoride was made and used by the fourteen students in the examination. To the surprise of the examiners the vast majority of the class found the fluoride to have no effect on the rate of salivary digestion. This negative result was confirmed by members of the laboratory staff at the time and it was decided to investigate the problem of differences between the action of the sodium and potassium salts. With three separate samples of sodium and potassium fluorides similar results were obtained; namely, no alteration of the time required to reach the achromic point with the sodium salt, and a very delayed action with the potassium salt. It was then thought advisable to see if the salts of the other halogens would show similar differences.

Experimental Method.

Normal solutions of NaCl, KCl, NH_4Cl, $CaCl_2$ and of the corresponding bromides, iodides and fluorides were made. In the case of calcium fluoride which is practically insoluble, a saturated solution was used, but results with this salt are necessarily unreliable owing to the minute amount in solution.

A 0·5 % solution of soluble starch in distilled water was also prepared, and some saliva collected directly into distilled water as described by Cole [1920, 1]. The solutions were warmed separately in a water-bath at 37°, and when at this temperature were mixed in the following proportions: 10 cc. starch; 2 cc. dist. water or salt soln.; 2 cc. saliva. The test-tube was then inverted to mix the contents, returned to the bath, and the time noted on a stop watch. At intervals of about 0·25 min. a drop of the digestion mixture was taken and added to a drop of weak iodine solution on a white tile. The achromic point was taken as occurring when the drop of digestion mixture and iodine and a drop of distilled water and iodine, placed side by side on the tile, were identical in colour. This was the end point taken in the experiments, and the time was noted on the stop watch.

Thus these experiments were carried out on saliva with its natural salt content, and not on ptyalin itself. The need for stressing this point was shown by Cole [1903]. It was thought better to use saliva as such, since it is impossible to obtain an absolutely pure ferment, and also because salivary digestion is normally carried out by saliva and not by a dialysed extract.

Experimental Results.

Chlorides.

Standard with water	(1)	8·0 mins.
	(2)	8·0 ,,
NaCl	(1)	3·75 ,,
	(2)	3·55 ,,
KCl	(1)	3·75 ,,
	(2)	3·75 ,,
NH_4Cl	(1)	4·80 ,,
	(2)	5·0 ,,
$CaCl_2$	(1)	3·75 ,,
	(2)	3·75 ,,

Hence all these chlorides, as previously shown by many workers, accelerated diastatic action.

Fluorides.

Standard with water	(1)	8·0 mins.
	(2)	8·2 ,,
NaF	(1)	8·5 ,,
	(2)	8·3 ,,
KF	(1)	120·0 ,,
	(2)	125·0 ,,
NH_4F	(1)	162·0 ,,
	(2)	180·0 ,,

This ammonium salt mixture seemed to be very acid, therefore ammonium hydroxide was added till it was markedly alkaline. The figures then were:

$NH_4F + NH_4OH$	(1)	56·0 mins.
	(2)	62·0 ,,
CaF_2	(1)	8·0 ,,
	(2)	8·3 ,,

Here there is a marked difference in the various fluorides, sodium fluoride being inert, whilst potassium and ammonium fluorides exert a marked slowing effect. As stated before, owing to its insolubility, the experiments with calcium fluoride are not comparable and are inconclusive.

Bromides.

Standard with water	(1)	8·0 mins.
	(2)	8·0 ,,
NaBr, KBr, NH_4Br, $CaBr_2$		8·0–8·2 mins.

Hence the bromides were without any action whatever on the rate of salivary digestion.

Iodides.

Standard with water ...	(1)	12·0 mins.
	(2)	12·0 "
NaI	(1)	12·0 "
	(2)	12·0 "
KI	(1)	12·25 "
	(2)	12·75 "
NH_4I	(1)	19·25 "
	(2)	20·0 "
CaI_2	(1)	44·0 "
	(2)	44·75 "

Here, as with the fluorides, there is a marked difference, the sodium and potassium salts being inert as regards effect on the rate of salivary digestion, whilst the ammonium and calcium salts delay it to a marked extent.

It was then thought that the differences might be due to variations in the acidity of the salt solutions since this factor is so important in physiological reactions, and the salt solutions were known to show large differences in their behaviour to indicators.

Accordingly the hydrogen ion concentration of these salts was estimated by Cole's comparator method [1920, 2] using phenol red as the indicator. The results are given in the following table:

Effect of salts on rate of Salivary Digestion.

Hastened action		Unaltered action		Delayed action	
NaCl... ...	p_H 7·0	NaF	p_H 7·6	NH_4I ...	p_H 5·6
KCl	6·6	*(CaF_2) ...	7·2	CaI_2	8·0
NH_4Cl ...	5·6	NaI	8·0	KF	7·8
$CaCl_2$... ...	7·2	KI	7·0	NH_4F ...	6·6
		NaBr ...	6·8	NH_4F+NH_4OH	8·0
		KBr	6·8		
		NH_4Br ...	5·8		
		$CaBr_2$ ...	8·0		

* Very insoluble, and therefore not really comparable.

From this table it appears that the chlorine ion exerts a definite accelerating action on salivary digestion rate, that the bromine ion is inert, and that the metallic ion is relatively unimportant in this connection.

With the fluorides and iodides there are marked differences, some of the salts delaying digestion, whilst others are inert. This difference does not seem to be due either to the metallic ion, or to the hydrogen ion concentration of the salt solution, but to be a definite effect of the salt as a whole, independent of the hydrogen ion concentration or the position of the ions in the periodic system.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XXXI. THE INFLUENCE OF DIFFERENT SUBSTANCES ON THE DIASTATIC ACTIVITY OF SALIVA.

By HILDA WALKER.

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(*Received February 20th, 1925.*)

This research was undertaken to determine the action of sweet substances on the diastatic activity of saliva, both as regards the secretion of ptyalin, and its activity when produced.

Recent work, which has a bearing on the subject, is as follows.

Iljin [1922] has found that salts accelerate the production of diastatic enzymes, probably by hastening the transition from the inactive zymogen to the active zymin. Rockwood [1918] showed that the ammonium salts of inorganic acids increased the activity of ptyalin, as did also those of the stronger organic acids. Groll [1918] showed that anions exert a greater influence than cations. The halogen ions increase and those of the sulphates and nitrates inhibit the activity of ptyalin. Ambard [1923] has found that starch adsorbs a definite amount of salivary amylase. Neilson and Lewis [1908] determined the effect of diet on the amylolytic power of saliva. They found that after feeding for some days on a carbohydrate diet the amylolytic power is increased, whilst it is decreased on a protein diet. Sanctis-Monaldi [1920] found that the secretion of parotid saliva was increased by various stimuli, such as bread, meat and acid, but its amylolytic power remained unchanged.

Method. Various substances were put into the mouth and rolled between the tongue and palate, and the activity of the saliva so secreted was compared with that of the saliva previously secreted in the empty mouth. The saliva was as a rule collected between 10 and 11 a.m., breakfast having been taken about 8 or 8.30. In each experiment the mouth was rinsed out three times with tap water and three drinks of water were taken; the saliva then collected was called *N*. After its collection the same procedure was gone through; the substance to be tested was put into the mouth and the saliva so produced called *S*. In each case the saliva was collected into a dry dish. 1 cc. was transferred to a 100 cc. flask, which was then filled to the mark with distilled water. The liquid was then filtered. Throughout the experiment the flasks, dishes and test-tubes were boiled before use.

The estimation of the diastatic activity was carried out by a modification of Wohlgemuth's method.

In a series of twelve test-tubes the diluted filtered saliva was added to a 0·9 % solution of sodium chloride according to the following table, each test-tube thus containing 1 cc. of liquid.

Test-tube	cc. diluted saliva	cc. 0·9 % NaCl
1	0·35	0·65
2	0·33	0·67
3	0·31	0·69
4	0·29	0·71
5	0·27	0·73
6	0·25	0·75
7	0·23	0·77
8	0·21	0·79
9	0·19	0·81
10	0·17	0·83
11	0·15	0·85
12	0·13	0·87

To each tube was then added 2 cc. of 0·1 % solution of starch. Soluble starch was used; the solution was free from chloride and neutral to litmus.

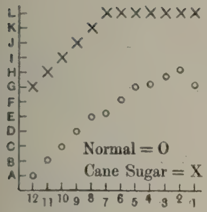
The set of tubes was then put into an incubator for 30 minutes. The temperature of incubation was as a rule 38°, but in various experiments there were slight variations above and below this.

After incubation the tubes were cooled by transference for 3 minutes to water at the temperature of the laboratory. To each tube was then added one drop of a *N*/50 solution of iodine.

Instead of taking the achromic point as a measure of diastatic activity it was found more convenient to make a series of standard colours ranging from blue through purple, red-purple, red-brown to yellow, and by comparison of the experimental colours with those of a standard, to plot a graph for the diastatic activity. The preparation of the standard colours was difficult, as it was not easy to match the exact tint, and when this was done, often after a few days it was found that a precipitate had appeared, or that the colour had faded. Mixtures of the following solutions were found to be most satisfactory: methylene blue, Uffelmann's reagent, magenta, carbol fuchsin and Bismarck brown. The exact strength and proportion of each solution cannot be given, as in each case it was a matter of repeated trial, adding first a little of one and then a little of another until a match was obtained to the colours produced by iodine on partially digested starch at different stages.

The colours were as follows:

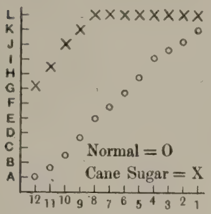
A = Blue ≡ no digestion.
B = Blue with a tinge of purple.
C = Purple.
D = Red-purple.
E = Magenta.
F = Red.
G = Pale red-brown.
H = Pale red-brown, but more brown and less red than *G*.
I = Pale brown with a tinge of red.
J = Paler brown with a tinge of red.
K = Yellow-brown.
L = Yellow ≡ complete digestion.



Subject H. W.

Graph 1.

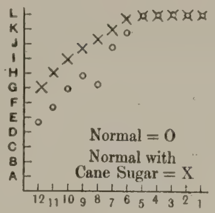
Cane Sugar



I. J.

Graph 2.

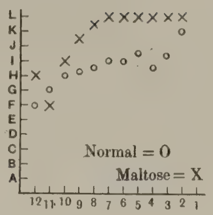
Cane Sugar



H. W.

Graph 3.

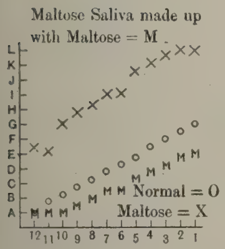
Normal Saliva made up with 10 per cent. Cane Sugar



H. W.

Graph 4.

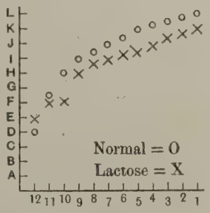
Maltose



W. M.

Graph 5.

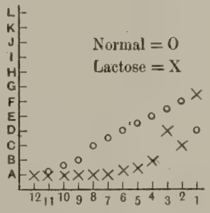
Maltose



H. W.

Graph 6.

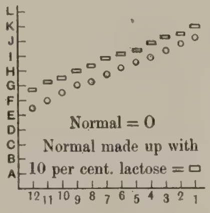
Lactose



D. P.

Graph 7.

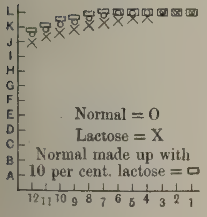
Lactose



H. W.

Graph 8.

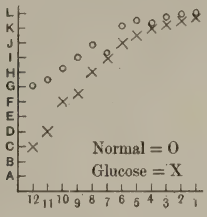
Lactose



H. W.

Graph 9.

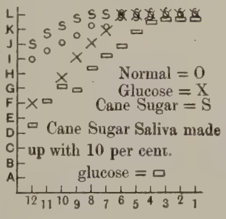
Lactose



H. W.

Graph 10.

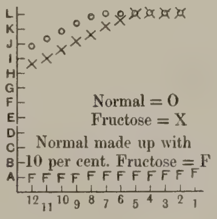
Glucose



H. W.

Graph 11.

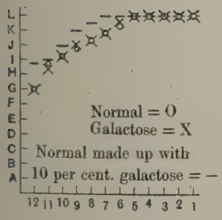
Glucose, Cane Sugar



H. W.

Graph 12.

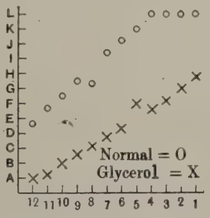
Fructose



H. W.

Graph 13.

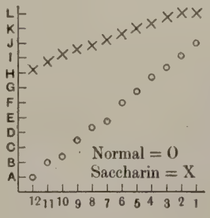
Galactose



H. W.

Graph 14.

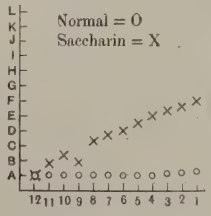
Glycerol



H. W.

Graph 15.

Saccharin



E. W. C.

Graph 16.

Saccharin

The numbers on the base lines are those of the series of tubes.

Since the experiments were comparative no determinations of the p_H were made. It is assumed that in each experiment it was the same for the normal and stimulated saliva, as the substances used were as far as possible pure. In any case the dilution was so great that a slight variation in the p_H would make very little difference.

In graph 1 it will be seen that the starch in the tube containing the smallest amount of normal saliva has undergone no digestion, while the corresponding tube of saliva after cane sugar shows considerable digestion. In each series the amount of digestion increases with increasing quantities of saliva, but in the *S* set digestion is complete in tube 7 and the following tubes, and presumably with a larger quantity of starch the curve would continue its upward direction, while in the *N* set, tube 2 with greatest digestion is only equivalent to tube 11 of the *S* set. A similar result is seen in graph 2.

From these graphs it is seen that cane sugar in the mouth has a very definite accelerator action on the saliva produced.

In order to determine whether this increase in activity was due to an increase in the quantity of ptyalin, or to an activating effect of the sugar upon it when produced, some normal saliva was collected in the usual way; of this 1 cc. was made up to 100 cc. with distilled water, and 1 cc. was made up to 100 cc. with 10 % cane sugar solution. The result is shown in graph 3.

From this it is seen that the presence of cane sugar increases the activity of ptyalin.

Maltose (graph 4) was found also to accelerate the action of saliva. In order to determine whether this was due to activation of the ptyalin, 1 cc. of the maltose saliva was made up to 100 cc. with 10 % maltose solution. The effect, as seen in graph 5, was to depress the action, and therefore the stimulating effect of maltose in the mouth must be due to a greater secretion of ptyalin.

The results of lactose in the mouth are shown in graphs 6 and 7; it appears to have an inhibitory action on saliva. In the next experiment 1 cc. of normal saliva was made up to 100 cc. with 10 % lactose solution, and the activity of this solution was compared with that of the normal saliva made up with saline in the usual way. The result is recorded in graph 8, from which it appears that the presence of lactose in solution enhances the activity of ptyalin. The result of a further confirmatory experiment is shown in graph 9.

Glucose in the mouth also has an inhibitory effect (graph 10).

In graph 11 the result of a comparison between normal, cane sugar, and glucose saliva is shown. The inhibitory effect of glucose was also determined by making up 1 cc. of the cane sugar saliva to 100 cc. with 10 % glucose solution. The graph shows the very marked inhibitory action of glucose.

Fructose in the mouth has a depressing effect, and that this is due mainly, if not entirely, to an inhibition of the ptyalin is seen from graph 12, which shows the result of making up 1 cc. of normal saliva with 10 % fructose solution, in which case there is practically no digestion at all.

Galactose seems to have very little effect, though its presence in solution slightly increases the activity of ptyalin (graph 13).

Glycerol (graph 14) in the mouth has a retarding influence. Saccharin, of which the sodium salt was used, has an activating effect (graphs 15 and 16).

SUMMARY.

From these experiments it is apparent that when various substances are taken into the mouth two effects have to be considered:

(1) an increase or decrease in the quantity, or possibly the quality, of the ptyalin secreted,

(2) a stimulating or depressing action on the enzyme produced by the substance being tested.

Of the substances examined, cane sugar and saccharin in the mouth appear to have the most marked effect in producing a very active saliva. In the case of cane sugar this is largely, if not entirely, due to its stimulating action on the ptyalin.

The behaviour of maltose and lactose is interesting; while maltose increases the quantity of ptyalin produced, its presence depresses the activity of the enzyme. Lactose, on the other hand, tends to inhibit the production of ptyalin, but enhances its activity.

Of the monosaccharides glucose and fructose both lower the activity of saliva, and in each case it is largely due to the depressing action of the sugar on ptyalin.

Further investigations on the same lines are proposed.

My best thanks are due to Professor Carlier (at whose suggestion the work was begun) for much helpful advice and criticism.

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XXXII. A COMPARISON OF DRIED AND EVAPORATED MILKS BY A DIETETIC METHOD.

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(*Received February 12th, 1925.*)

At the present time there are a great number of dried and evaporated milks on the market, and many of them appear to have been used successfully in infant feeding. In fact, some makes are regarded as superior to fresh cow's milk, in that there is no possibility of the milks turning sour. Dried milks are made by one of two methods, either the "roller" or the "spraying" process being used. There seems to be no conclusive evidence as to which method supplies the best results, except with regard to vitamin C, when less destruction is claimed with the "roller" process [Jephcott and Bacharach, 1921; Hart, Steenbock and Ellis, 1921]. Experimental work on this subject has been done by various observers, a survey of the literature being given in the report of the Medical Research Council [1924].

The experiments to be described in this paper were started in the hope of obtaining information as to the vitamin B content of dried and evaporated milks. It had been found previously [Hartwell, 1921] that an excess of protein in the lactating rat's diet resulted in the offspring's suffering from spasms, etc., and that the young rarely survived. Later [Hartwell, 1922] it was shown that the addition of 100 cc. of whole cow's milk to the mother's excess protein diet entirely prevented the abnormal symptoms in the sucklings. Therefore it seemed possible that this fact might be utilised as a basis for comparison of different kinds of dried and evaporated milks.

Experimental.

1. *General Principle of Method.*

A diet was chosen which had been found to cause death of the offspring, while the mother remained fit and well. To this basal diet were added different quantities of fresh cow's milk in order to find the least amount necessary to protect the young from spasms. Dried or evaporated milks were then substituted in turn for the fresh cow's milk and the results compared.

2. *Diets.*

The basal diet consisted of bread and food casein [Hartwell, 1922] fed in the proportion of 15 g. bread to 6 g. food casein. To this basal diet were added successively 75, 50, 33, 24, 18 and 12 cc. of fresh cow's milk, water being added when necessary to make the mixture into a paste.

(*a*) *For the dried milks* the following proportions were used:

Dried milk, full cream,	12 g.	to 100 cc. water.
,, ,, half ,,	10·5 g.	,, 100 ,, ,,
,, ,, skim,	9 g.	,, 100 ,, ,,

These amounts were used in place of 100 cc. of cow's milk. They were chosen in accordance with the directions on the tin, the object being to use the same amount of powder as would be taken by the average person making up the milk from the given directions. Therefore different people were asked to measure out the amount stated on the tin and these amounts were weighed. For example, take x tablespoonfuls of dried milk to y pints of water. Twenty people were asked to measure x tablespoonfuls; these different quantities were then weighed and the average obtained. This amount was used with y pints (converted to cc.) of water. It is interesting that the figure obtained in this way is near the average of 12·4 % given for total solids in milk [Plimmer, 1921], but, of course, using this proportion does not allow for any water in the dried milk.

(*b*) *For evaporated milks*, the dilution used was that given on the tin, and this diluted milk was substituted for cow's milk. The specific gravity of the liquid was approximately that of fresh cow's milk.

The control mothers were fed on bread and milk, 15 g. bread to 50 cc. cow's milk (or its equivalent).

3. *Method.*

At least three mother rats were given each diet, and they received food *ad lib.* The mother and young were weighed daily. The babies were watched carefully for any abnormal symptoms. The mother rats had a good mixed diet during gestation and only litters of good weight at birth were used in these experiments. The special diet was given as soon as possible after the birth of the litter. In all, 171 mother rats and litters with 6 young to a litter were used in these experiments.

RESULTS.

The results are given in the Table, p. 228. In every case the condition of the young became worse as the mothers received smaller proportions of milk (Fig. 1, cow's milk is given as illustration). It is obvious that fresh and dried milks much excel the evaporated milks in vitamin B content. For example, it is significant that 75 cc. of diluted evaporated milk are insufficient to prevent spasms in the young, while 33 cc. of cow's milk or the equivalent amount of some dried milks are almost sufficient, no spasms and only slight

Proportions of Milk added to Basal Diet of Mother Rat.

Sample	Control	I. 75 cc.	II. 50 cc.	III. 33 cc.	IV. 24 cc.	V. 18 cc.	VI. 12 cc.	Remarks
Cow's	50 cc. milk to 15 g. bread	Normal condition. Growth curve depressed	Same as I	Growth about the same as in I, but animals not quite normal, slight incoordination shown. 1 out of 18 died	Growth more depressed than in I, II and III. All 3 litters showed spasms and 1 litter had screaming fits	Growth similar to IV, but curve flattened from 15th day, some loss of weight. All 3 litters had spasms and screaming fits. 1 out of 18 died	Condition of young very bad from 12th day. All 3 litters showed typical symptoms and 2 whole litters died, *i.e.* 12 out of 18	—
Dried.								
A ‘roller”)	Rate of growth of sucklings equal to that obtained with cow's milk	—	Same as with cow's milk	Growth not quite so good as in II. Slight spasms shown. 1 out of 18 died	Growth about same as III. All 3 litters showed incoordination. 1 litter had spasms and screaming fits. None died	Growth similar to III. 2 litters spasms and 1 litter screaming fits. 1 out of 18 died	Growth slower but 2 litters successfully weaned, although all 3 litters abnormal. Two young died	Slightly better than cow's milk
B (“roller”)	Do.	—	Same as with cow's milk, but slight incoordination in 1 litter. 1 baby died	Same as II	Growth about the same as II, but all 3 litters had spasms and screaming fits. 1 baby died	Growth and condition very poor, 2 whole litters and 2 other young died, *i.e.* 14 out of 18 died	Condition very bad from 12th day. 1 whole litter died, and 5 from each of the other litters, *i.e.* 16 died	Slightly worse than cow's milk
C (“roller”)	Do.	—	Same as II B	Growth depressed, all 3 litters spasms; 1 litter did not gain weight after the 14th day	Similar to III	Condition of young poor. 2 whole litters died, the other showed no gain after the 15th day	Condition very bad but 7 survived	Slightly worse than cow's milk and similar to sample B
D 1 (“roller”) (full cream)	Do.	—	Growth depressed, 2 litters had spasms, and in addition had screaming fits, *i.e.* only 1 litter was normal	Growth same as II. All 3 litters had spasms, and 1 litter screaming fits	Growth depressed and curves very flattened from 14th day. All 3 litters had bad spasms and 2 had screaming fits. 1 out of 18 died	Condition very bad, all 3 litters died	—	Worse than cow's milk
D 2 (“roller”) (half cream)	Not quite so good as D full cream	—	Very poor growth; all 3 litters had spasms and screaming fits. 5 died	Same as II, but only 1 died	Same as III, only 1 died	—	—	Not quite so good as D full cream

E ("spray") (full cream)	Normal	—	All 3 litters had spasms and 3 young died	—	—	All 3 litters had spasms and 1 whole litter died	—	About the same as D full cream
F 1 (full cream) ("spray")	Rate of growth of sucklings equal to that obtained with cow's milk	—	Growth quite good, condition normal	Same as II; no spasms	—	All 3 litters had spasms and screaming fits. Growth curve flattened from 14th day. None died	All litters had spasms. 1 whole litter and 2 other babies died, *i.e.* 8 died	Slightly better than cow's milk; equal to A
F 2 (skim) ("spray")	Almost maximal growth	—	Growth good, but not up to standard. No spasms	Growth good. 2 litters had spasms and all 3 showed incoordination	—	Growth curve very depressed, all 3 litters spasms. 1 whole litter died	—	Not so good as F 1
Evaporated.								
G	Rate of growth a little slower than with cow's milk; young rats had thin coats and were very limp to the touch	Growth curve depressed. All 3 litters exhibited incoordination and 2 had spasms. 1 baby died	Same as I, but condition of young just worse. All 3 litters had spasms and screaming fits. 1 died	Very slow growth of young. All 3 litters showed spasms and screaming fits. 1 died	Same as III, but 2 died	Condition worse than IV, 1 whole litter and 2 other young died	—	Not quite so good as D 1, distinctly poorer than cow's milk
H	Similar to G	—	Growth very poor indeed, 2 litters ceased to gain weight from the 14th day. All 3 litters had spasms and screaming fits. 2 died	Condition similar to 2, but worse. 9 died	Similar to III. 8 died	Condition worse than IV. 12 died	—	Not quite so good as G
I	Similar to G and H	—	Growth curve depressed. 1 litter had bad spasms. 5 died	—	All 3 litters had bad spasms and screaming fits. 1 whole litter died and 3 other babies	—	—	—
J*	—	Growth fairly good, but 1 litter had spasms and 1 litter screaming fits	—	—	—	—	—	—

* It was impossible to obtain any more of sample J.

incoordination being noted. A comparison of some of the samples is shown in Fig. 2.

The control experiments (where the mother rats were fed on bread and milk alone, *i.e.* no extra protein) also are interesting, in that they support

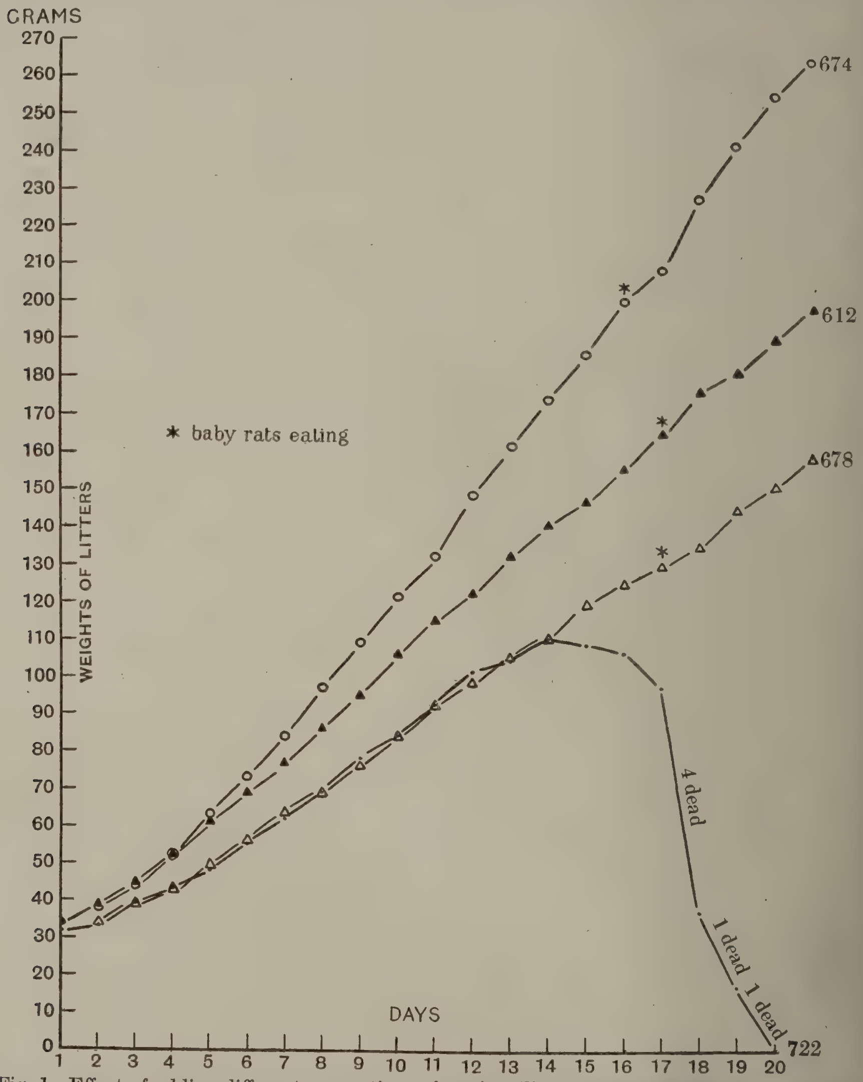

Fig. 1. Effect of adding different proportions of cow's milk to a basal diet of "excess" protein.

Mothers' diets.

○——○ 674. 15 g. bread, 50 cc. cow's milk. Control.
▲——▲ 612. 15 ,, ,, 6 g. food casein, 50 cc. cow's milk.
△——△ 678. 15 ,, ,, 6 ,, ,, ,, 24 ,, ,, ,,
•——• 722. 15 ,, ,, 6 ,, ,, ,, 12 ,, ,, ,,

the general view that evaporated milks are not so good as the dried varieties. The young belonging to the mothers fed with dried milk and bread were normal, and appeared equal in all respects to the young whose mothers had bread and fresh cow's milk. The young whose mothers received evaporated milks were by no means up to standard. Their weights certainly were quite good, though slightly below normal, but their coats were poor and the baby

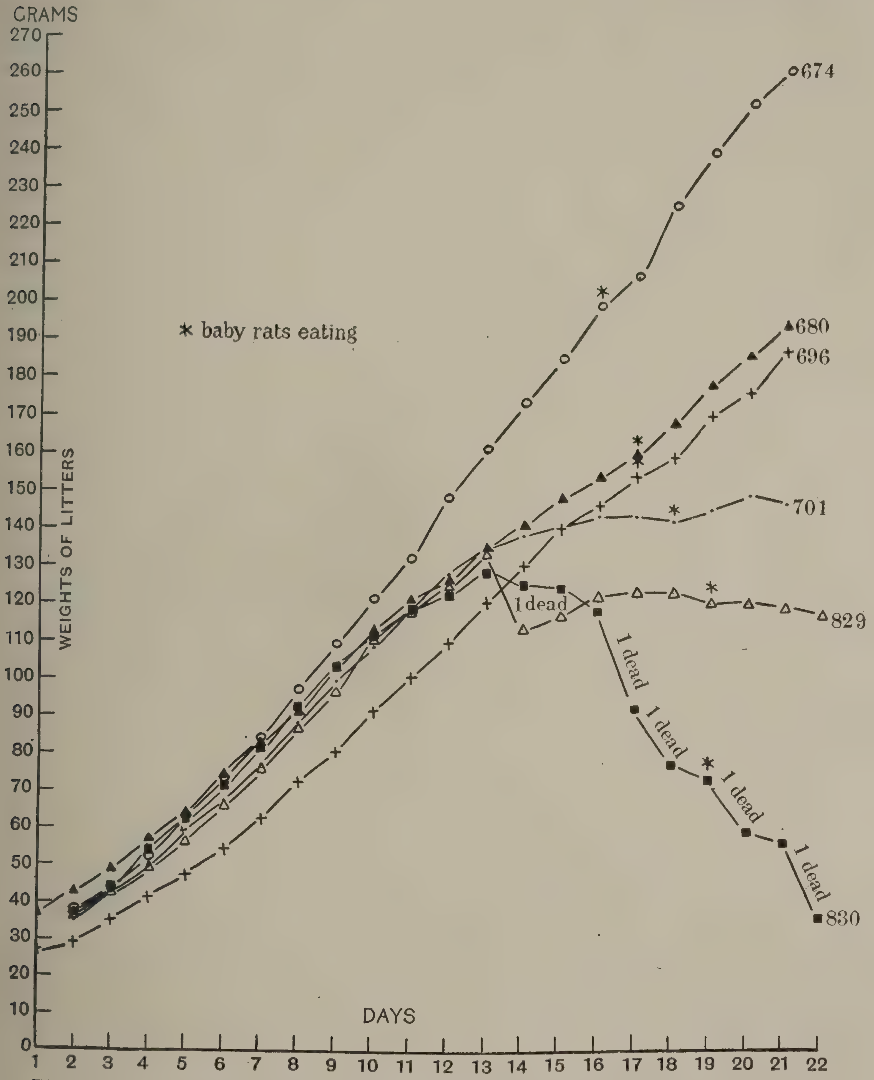

Fig. 2. Effect of adding equivalent amounts of cow's, dried and evaporated milks to a basal diet of "excess" protein.

Mothers' diets.

○——○ 674. Bread and cow's milk. Control.
▲——▲ 680. Basal diet + cow's milk.
+——+ 696. ,, ,, + dried milk; sample A.
•——• 701. ,, ,, + ,, milk; ,, D 1.
△——△ 829. ,, ,, + evaporated milk; sample G.
■——■ 830. ,, ,, + ,, milk; ,, H.

rats were limp to the feel. In other words they were just not fit. Confirmatory results were obtained in a series of experiments, to be published shortly, on the growth of weanling rats on dried, evaporated and fresh cow's milk. Summing up one can say that samples A and F1 (dried milks) are just better than cow's milk, and samples B and C (also dried milks) just less good, while samples G, H, I and J (evaporated milks) are distinctly inferior as compared with cow's milk in regard to vitamin B content.

Comments and Conclusions.

The evidence is quite conclusive that evaporated milks are poorer in vitamin B content than is fresh cow's milk (heated to 70°). With dried milks on the other hand, there is no definite evidence as to which method ("spray" or "roller" process) produces the better results, since sample F1 ("spray") is quite as good as sample A ("roller"), while sample E ("spray") is as poor as sample D1 ("roller"). It is quite possible that some individual differences in manufacture are responsible for these variations.

As pointed out by Heineman [1919] dried milks prepared by the "spray" process are more easily soluble in water than are those prepared by the "roller" process. Also the former are easily clotted by rennet, *e.g.* sample E clots in approximately the same time as cow's milk, while a true junket is not obtainable from samples A and B. It may be that the manufacturing method most suitable for preserving the physical properties of milk is one which results in a greater destruction of vitamin B, and that a method safeguarding vitamin B results in an alteration in the physical condition of the proteins, etc., of the milk. It would be interesting to know if vitamin A suffers any similar destruction.

Summary.

1. A dietetic method is described for the comparison of cow's, dried and evaporated milks as regards vitamin B content.

2. The vitamin B content of evaporated milks is lower than that of dried milks and of fresh milk.

3. Dried milks vary in their vitamin B content, which may be slightly greater or slightly less than that of fresh milk. This variation does not run parallel with the method of manufacture.

4. Dried skim and half-cream milks are not quite so good as the same makes in full-cream.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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XXXIII. MERCURIALIS.

I. THE DEVELOPMENT OF A BLUE PIGMENT ON DRYING.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department of University College, London.

(*Received February 25th, 1925.*)

It has long been known that the green shoots of *Mercurialis perennis* and *M. annua* acquire a bluish hue on drying, a fact that is perhaps not as widely known to botanists as might be expected. The only reference to the nature of the blue pigment which we have been able to trace is one by Molisch [1893], who, in the course of a systematic search for indigo-producing plants examined *M. perennis* and dismissed this plant with the remark that the pigment is not indigo, since it is turned red by the addition of acid.

With regard to the mechanism of the production of the blue colour the possibility of enzyme action at once suggests itself since, in a great many cases, the post-mortem coloration of vegetable tissue has been shown to be due to the presence either of an oxidase acting upon some constituent of the substrate or of a hydrolytic enzyme acting upon a glucoside, as has been shown by Bridel and Braecke [1922, Bridel, 1923] for *Melampyrum*, *Monotropa* and other plants.

In the present instance of *Mercurialis*, however, it would appear from our observations that enzyme action of any kind is highly improbable. The possibility of oxidase action is excluded since this plant does not contain a direct acting oxidase but only a peroxidase which is incapable of exerting its activity until after the addition of a peroxide. The possibility of other enzyme action is unlikely in view of the fact that the blue pigment can be produced by drying the tissues after such treatment as immersion in boiling water or suspension in steam; further the pigment may be produced by leaving the tissues to soak for some time in absolute alcohol, a reagent which is not generally regarded as favourable for enzyme action. Our observations lead us to conclude that desiccation is the prime factor concerned in the production of this blue pigment.

The Preparation of the Blue Pigment by Desiccation.

(*a*) *By the use of absolute alcohol.* This reagent, as is well known, rapidly removes water from fresh tissues and also quickly expels the air contained within the intercellular space system of the plant, a point of some importance in view of the formation of another blue pigment by oxidation. The use of absolute alcohol enables one either to extract the blue pigment or, by slightly modifying the conditions, to bring about its deposition within the tissues. If the former be desired it is necessary to use the colourless rhizomes in order to avoid contamination with chlorophyll. The freshly-gathered rhizomes are washed free from adhering soil, dried with a cloth, rapidly cut up into short lengths and dropped into absolute alcohol contained in a flask; the latter is then gently warmed over a water-bath to a temperature not exceeding 40°. The resulting deep blue solution is filtered into a distillation flask and evaporated to small bulk under reduced pressure in a stream of nitrogen. The residual liquid consists of a violet coloured aqueous solution in which is suspended a flocculent greyish white substance, which on filtration and exposure to air darkens. This material contains phosphorus, and from its solubilities and general behaviour, appears to be of a lecithin-like nature, but it has not as yet been fully investigated. The aqueous filtrate on evaporation to dryness yields a dark blue material which sets to a brittle varnish.

If it is desired to produce the pigment in the tissues and to leave it there deposited with a view to studying its anatomical distribution, advantage may be taken of the circumstance that the pigment is insoluble in absolute alcohol and only comes out into solution if the alcohol is allowed to become diluted by the moisture withdrawn from the tissues. To study the distribution in green leaves or shoots it is therefore necessary to immerse them in absolute alcohol and to replace the alcohol frequently, as it takes up moisture from the tissues; in these circumstances only the chlorophyll goes into solution and the alcohol is accordingly coloured green and not blue. If such an experiment is carried out successfully the tissues gradually lose all their chlorophyll and assume an intense azure blue colour. The blue pigment can be removed from such tissues by immersion in water.

(*b*) *By drying.* In the early phases of this investigation it was observed that on drying the material developed the blue pigment very irregularly. In order to discover the best method, green shoots and leaves and colourless rhizomes were dried under a great variety of conditions, as a result of which it was found that the best yield of blue is obtained by drying as slowly as possible, in a dark cool place. Under these conditions of slow drying there is less tendency for the production of the unstable and readily oxidisable blue compound to be described later; the choice of a dark place is to be recommended more particularly in the case of green material, since there is less danger of contamination with possible degradation products of chlorophyll.

To extract the pigment from the tissues, the dried material is ground in a mill and a quantity of the resulting powder is stirred into cold water until the powder is thoroughly wet. The pigment rapidly diffuses out and the extraction is complete in half an hour, if the paste is kept well stirred. The material is then placed in a linen bag and expressed in a tincture press. The greenish-blue liquid thus obtained may be used for extracting a fresh quantity of the powder and by so doing a strong solution results. This is centrifuged and, if necessary, filtered.

Reference has already been made to the production, by oxidation, of a second blue compound which on further oxidation gives a yellow one. There is good reason to infer, as will be seen later, that both these blue compounds are produced from the same mother substance and that if the chromogen is converted into the transient blue compound it cannot form the permanent blue substance.

It is the production of the yellow substance, in varying amount, which is responsible for the fact that in a length of dried rhizome a portion may be yellow while the rest is peacock blue. The yellow compound being likewise soluble in water is extracted together with the permanent blue and is thus responsible for the peacock green colour of the extract. To remove this impurity, the centrifuged solution is saturated with ammonium sulphate, whereby the blue compound is precipitated; the filtrate, which contains the yellow compound together with a small additional quantity of the blue, is bright green in colour; on prolonged standing a little more of the blue compound is thrown out. The precipitated pigment is filtered off, washed with saturated ammonium sulphate solution and dried at 37°. The chemical properties of this substance are still under investigation.

Distribution of the Blue Pigment.

All parts of the dried plant, aerial shoots and subterranean rhizomes and roots, may show a peacock blue coloration, which appears in the last phases of desiccation, the colour being densest in the younger parts. Thus the apex of the shoot contains more pigment than the basal region, young leaves contain more than old, young white rhizomes are much richer than the older brown. In axial structures the pigment is restricted to the cortical parenchyma and in the leaves it is distributed more or less evenly throughout the parenchyma of the mesophyll.

Summary.

1. There exists in *Mercurialis* a chromogen which gives rise to a blue pigment on drying.
2. The production of the blue colour is not due to enzyme action.
3. Methods are described for the isolation of the pigment.
4. The yield of the pigment indicates that the chromogen is most abundant in the youngest and vigorously growing parts.

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XXXIV. MERCURIALIS.

II. THE OCCURRENCE OF A CHROMOGEN SHOWING A REMARKABLE AVIDITY FOR FREE OXYGEN.

By PAUL HAAS and THOMAS GEORGE HILL.

From the Botanical Department of University College, London.

(*Received February 25th, 1925.*)

With the object of extracting from *Mercurialis* the precursor of the permanent blue compound whose isolation is described in the previous paper [Haas and Hill, 1925], fresh tissues were closely packed into a glass cylinder provided with a glass stopper; the tissues were then completely covered with 10 % alcohol, the cylinder being filled to the top and the stopper inserted. On removing the stopper after 48 hours and withdrawing the tissues, the aqueous extract was found to be colourless, but after a few minutes the superficial layer assumed a blue colour which slowly disappeared on further exposure to air. These facts indicate that *Mercurialis* contains a chromogen which by oxidation yields a transient blue compound distinct from the permanent blue compound mentioned above. In view of the sensitivity of this chromogen to atmospheric oxidation, the following method of preparation was adopted.

Exp. 1. Cleaned white rhizomes or apical regions of young aerial shoots are cut into short lengths, the severed portions being allowed to fall into a wide-mouthed glass bottle containing just enough cold freshly-boiled water to cover the fragments of tissue used. Roughly 100 g. of rhizome cut into quarter-inch lengths require about 100 cc. of liquid, whilst the same weight of aerial shoot will need about 175 cc. of water. About 2·5 cc. of chloroform for each 100 cc. of water are then poured in and a rubber cork, fitted with inlet and outlet tubes, is rapidly placed in position, and a rapid stream of nitrogen is passed through in order to remove the air. After a few minutes the nitrogen stream is moderated and the bottle is placed in a thermostat at 45° for one hour.

If the experiment is carefully conducted the liquid in the bottle will be colourless, but on removal and filtration a striking change takes place; the extract rapidly alters in colour to a deep azure blue which may be preserved for some time if the solution be kept in an atmosphere of nitrogen. If, however, the extract be fully exposed to air, the blue increases to a maximum and then changes through green to yellow, the latter being permanent; the green phase is due to a mixture of the yellow with the blue. The depth of colour developed varies with the strength of the solution; thus from vigorous young shoots the blue pigment may be so deep as to appear almost black and this, on complete oxidation, will give a brown colour. The residual tissues remaining after extraction are no longer able to form the permanent blue on drying.

Another way of demonstrating the colour changes resulting from atmospheric oxidation is to fill a glass cylinder with the blue solution and to leave it open to the air. In time the surface layer will change to yellow, so that a sharp line of demarcation is formed between the yellow and the blue. This line moves slowly downwards as the oxidation of the blue compound proceeds. The sharp separation between the yellow column above and the blue column below is easily broken, wherefore the greatest care must be taken to avoid shaking and the formation of convection currents. This also serves to demonstrate the rate of solution and diffusion of oxygen into the solution and it is possible that this pigment may find application in the investigation of gaseous diffusion, not only in liquids but also in gels.

A similar cycle of changes in colour can also be produced in the tissues by cutting, bruising or otherwise injuring them. Thus if a fresh piece of white rhizome is cut across with a sharp knife, the cortical layer will in a short time assume a blue colour which gradually fades away. Or again, if a small quantity of rhizome is crushed in a white porcelain dish the material quickly assumes a blue colour which changes to dull green and finally to yellow. If the tissue has been well crushed and frequently turned over to ensure thorough exposure to air the pulp will no longer give rise to the permanent blue on desiccation. Similarly it is found that exposure to chloroform vapour induces in a colourless rhizome the formation of the transient blue, the oxygen in this case being presumably supplied by the air contained in the intercellular space system of the tissues. Here again it is found that a portion of the tissue which has been exposed to chloroform vapour for a sufficient length of time to ensure the maximum formation of the transient blue and its subsequent oxidation, will no longer yield the permanent blue on drying. This may be conclusively shown by passing a long rhizome through a cork into a test-tube containing a little chloroform, in such a way that part of the rhizome is within the tube and the part on the other side of the cork is exposed to the air. The enclosed portion will soon turn blue and next day it will be yellowish white, whilst the part exposed to the air will be blue, owing to the formation of the permanent blue.

Exp. 2. That the changes here described are due solely to direct atmospheric oxidation without the intervention of an enzyme was shown as follows. Young white rhizomes were extracted with chloroform water in an atmosphere of nitrogen as described above. The vessel was connected by a rubber tube to a distillation flask through which nitrogen also circulated. After warming for an hour at 45° the extract was transferred through the rubber tube from the extracting bottle to the distillation flask without exposure to air. The extract was then boiled vigorously over a naked flame for one minute in a current of nitrogen and, after cooling, exposed to air. The characteristic colour changes from colourless through blue and green to yellow were obtained, showing that the phenomenon is able to take place under circumstances which preclude the possibility of enzyme action.

It has been shown that a permanent blue compound is formed by *Mercurialis* on drying [Haas and Hill, 1925]; it is now seen that the plant also yields a transient blue compound by atmospheric oxidation. The question arises whether the same chromogen is involved in each phenomenon—a precursor which by loss of water yields the permanent blue and by oxidation gives the fugitive blue. To test this the following experiment was carried out to see whether the chromogen extract, when evaporated to dryness in an atmosphere of nitrogen, could be made to yield the permanent blue.

Exp. 3. A chloroform water extract was transferred to a distillation flask as described in Exp. 2 and there evaporated to dryness under reduced pressure in a current of nitrogen. The flask contained a small quantity of a deep blue solid which, when taken up with water, and exposed to the air retained its colour unimpaired, from which it may be concluded that the conversion of the chromogen into the permanent blue had been effected by desiccation, as had been anticipated.

It will be remembered that tissues which have produced the transient blue to their fullest extent never yield the permanent blue on desiccation (p. 237). For these reasons we incline to the view that it is the same chromogen which yields either the one or the other blue compound according to the conditions, but a sufficient answer to this question cannot be given until the chemistry of the substances involved has been fully investigated.

The possible physiological significance of such an extremely labile substance as the chromogen described is of the greatest interest, but speculation at this early stage is unprofitable; much more information is requisite. Thus, before considering this substance as a respiratory chromogen in Palladin's sense, but differing from his in that enzyme action is not involved, it is necessary to ascertain whether the oxidative changes producing the blue and yellow compounds are reversible or not. In answer to this question it may be said that so far only the oxidation of the chromogen to blue has been reversed; thus, if to a solution of the fugitive blue a little sodium hydrosulphite is added the blue colour is discharged, but is restored by careful addition of hydrogen peroxide, only to be further and irreversibly oxidised to the yellow compound on the addition of excess of the reagent. Attempts to reduce the yellow compound back either to the blue or to the chromogen by means of the same or other reagents have so far not met with any success and solutions of the yellow compound treated with excess of hydrosulphite give no indication of production of the blue on carefully regulated addition of hydrogen peroxide.

These and other aspects of the subject are under investigation and our observations, together with some account of the chemical properties of the substances involved, will form the subject of a future communication.

SUMMARY.

1. In *Mercurialis* there is a chromogen which undergoes oxidation by gaseous oxygen in two stages, the first stage results in the production of fugitive blue compound which is further oxidised to a permanent yellow substance.

2. The oxidation of the chromogen is independent of enzyme action.

3. The fugitive blue is distinct from the blue produced on desiccation.

4. There are reasons for considering that there is only one chromogen concerned, which forms either the fugitive blue or the permanent blue, according to conditions.

We desire to express our indebtedness to Miss Beatrice Lee, M.Sc., for her valuable help in the preliminary phases of the investigations described in this and the previous paper, whilst working in this laboratory under a grant from the Department of Scientific and Industrial Research.

REFERENCE.

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XXXV. SYNTHESIS OF VITAMIN A BY A FRESH-WATER ALGA, *CHLORELLA* (sp.?).

By KATHARINE HOPE COWARD.

From the Biochemical Department, University College, London.

(*Received February 26th, 1925.*)

Jameson, Drummond and Coward [1922] showed that a marine diatom, *Nitzschia closterium* W. Sm., synthesises vitamin A when grown in Miquel's solution or sterilised sea-water in diffused sunlight. This work was, however, open to the criticism that the culture, one supplied by Dr Allen, Plymouth, was not pure, having been found to contain bacteria. Hence it was decided to repeat the work on a culture of *Chlorella* (species undetermined) which was known to be free from bacteria.

Two experiments were performed. In each the culture medium consisted of:

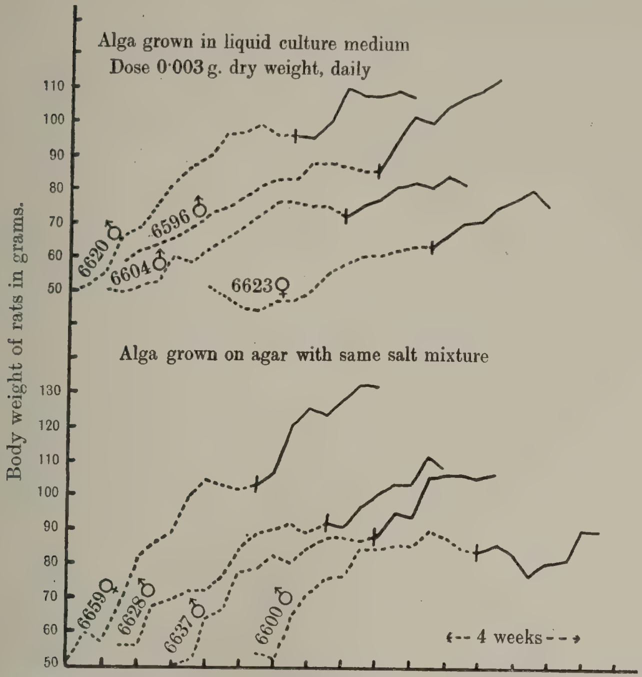
$Ca(NO_3)_2, 4H_2O$	1·7 g.	dissolved in water,
$MgSO_4, 7H_2O$	0·41 g.	
$CaCl_2$ (anhydrous)	0·123 g.	
$FeCl_3$	50 cc. 0·005 % solution	
K_2HPO_4	0·20 g. dissolved separately.	

The whole of the above was made up to 1000 cc.

For one experiment the above solution was used alone; for the other, 1·5 % agar was added to it. Four flasks of 250 cc. capacity and containing 30 cc. of the culture medium were inoculated daily in each experiment with an emulsion of the alga, for thirty days. Bacteriological precautions were used throughout. The flasks were plugged with cotton-wool, covered with caps of paper and kept near a window with a south aspect for two months in the late summer. In no case were any signs of bacterial growth observed.

For the feeding test the culture was filtered through a wad of starch in a Gooch crucible, as in the previous experiment on *Nitzschia*. In the case of the cultures on agar, the alga was scraped off, and mixed intimately with a small piece of diet. The daily dose per rat (the culture of one flask) was approximately 0·003 g. dry weight. The basal diet, free from vitamin A, was the one used in the diatom experiment and in general use in these laboratories.

Marked resumption of growth in the test animals indicated that *Chlorella* (sp.?) grown in pure culture had synthesised vitamin A.



Preparatory period on basal diet free from vitamin A shown in dotted lines

The thanks of the writer are due to Dr Roach of the Rothamsted Experimental Station for supplying the culture of *Chlorella*, and to the Medical Research Council for a grant from which the expenses of this experiment were defrayed.

REFERENCE.

Jameson, Drummond and Coward (1922). *Biochem. J.* **16**, 482.

XXXVI. THE PHOSPHATE METABOLISM IN FATIGUED MAMMALIAN MUSCLE.

By SAMUEL ANDREWS.

From the J. C. White Department of Biochemistry and the Physiology Department, Queen's University, Belfast.

(*Received February 27th, 1925.*)

The extreme importance of the changes undergone by the hexosephosphoric esters in the metabolism of muscle has only within recent years been recognised. In a long and important series of papers from Embden and his school the general nature of the changes in these esters (lactacidogen) has been gradually elucidated.

The influence exerted by the various anions on the metabolic changes, anabolic and catabolic, which lactacidogen undergoes, has been the subject of certain recent researches by Embden and Lehnartz [1924], Embden and Haymann [1924], Abraham and Kahn [1924], and Lange and Meyer [1924]. From these investigations it is evident that various anions, such as thiocyanate, chloride, etc., give rise to a disintegration of the lactacidogen molecule, whilst others, especially the fluoride and oxalate, produce a synthesis. From Embden and Haymann's work it is evident that the action of the fluoride is a true synthetic one, because accompanying the disappearance of the free phosphoric acid there is a definite formation of the diphosphoric ester. It is also evident that the process does not require the intact muscle fibre, as it is to be observed in the expressed muscle juice as well as in minced muscle.

It is extremely difficult in many cases, as Embden, Schmitz and Meincke [1921] have proved for frog's muscle, and to a lesser extent for dog's muscle, to show that the lactacidogen diminishes during activity, owing to the rapidity with which a recuperative synthesis of the carbohydrate and the phosphate takes place.

In this communication attention will be directed mainly to the different effects produced by the fluoride anion acting upon resting and stimulated muscle. It was recognised from Embden's investigations and also from many of my own, that this synthetic action might be increased by the addition of glycogen to the fluoride solution; but the effects produced by the fluoride alone are so well marked that such an addition was found to be unnecessary.

Embden, when estimating the amount of phosphoric acid already preformed in the muscle, made use of a method in which he fixed the muscle in hydrochloric acid and then precipitated the protein with mercuric chloride, removing the excess of mercury with sulphuretted hydrogen. The value of free phosphate which he found in his filtrate he called the A value. Then he warmed the muscle with sodium bicarbonate solution in order to break down the lactacidogen. The total phosphate value thus obtained he called the B value. The difference between B and A was the amount of phosphoric acid bound as ester.

In the experiments which follow I shall make use of these letters when referring to the free and total phosphate values, and the value after the action of fluoride I shall refer to as the C value.

The resting and stimulated muscle of the rabbit, cat and dog were examined. In all the experiments on rabbit muscle the pale variety was used.

TECHNIQUE.

The muscle, immediately it was taken out, was placed in a vessel cooled in ice. Small specimens (about 1 g.) were quickly minced on an ice cold porcelain slab, and placed in 5 cc. quantities of the various solutions. The protein was precipitated by the addition of trichloroacetic acid, and the muscle was finely ground up in a mortar and filtered. The extraction was repeated three times and the washings all passed through the same filter.

The inorganic phosphate was estimated in the filtrate by Embden's gravimetric method [1921], which was found in all the controls to give very accurate results. The preformed inorganic phosphate (Embden's A value) was found by taking the muscle directly into 5 % trichloroacetic acid. As will be seen, this method gives results very comparable with those obtained by the hydrochloric acid extraction method. The lactacidogen values (Embden's B – A value) were obtained by heating the muscle with 2 % sodium bicarbonate for 2 hours at 40° in each case.

Numerous controls were carried out, and duplicates were done with the majority of the specimens. It was found that the duplicates, when the conditions were similar, did not differ by more than 2–3 %.

RABBIT MUSCLE.

In the first three experiments resting rabbit's muscle was examined. The animals had been fed for 2 days previously with oats and carrots. Each was killed by a blow on the back of the head, and bled. Portions of pale muscle were quickly excised from the adductors of the lower limb.

	% H_3PO_4		
	Rabbit I	Rabbit II	Rabbit III
In 5 % trichloroacetic acid	0·356	0·332	0·373
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·576	0·601	0·623
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·522	0·550	0·546
In *N*/10 sodium fluoride, 2 hours	0·093	—	—
3 ,, ...	—	0·057	0·100
5 ,, ...	—	0·057	—
6 ,, ...	0·044	—	—
18 ,, ...	0·118	—	—
heated 2 ,, ...	0·107	—	—
In *N*/10 sodium fluoride + 0·4 % glycogen, 3 hours 12°	—	0·012	0·057

If we take the trichloroacetic acid value as corresponding to Embden's A value and the heated bicarbonate figure as corresponding to his B value, we obtain in each case the lactacidogen value.

	Rabbit I	Rabbit II	Rabbit III
Lactacidogen	0·220 %	0·269 %	0·250 %

The preformed inorganic phosphate value does not vary much in the three animals.

On heating with bicarbonate there is a marked rise in the inorganic phosphate content, corresponding to increases of I, 61·8 %, II, 81·0 %, III, 67·0 %.

On heating with normal saline, the chloride ion is also found to exercise a disintegrative effect on the lactacidogen, although the increase is never so marked as with bicarbonate. Increases: I, 46·6 %, II, 65·6 %, III, 46·4 %.

Under the action of fluoride there is a marked decrease in the amount of inorganic phosphate; this action seems to reach its maximum in 3–5 hours.

The differences in the trichloroacetic acid value and the fluoride value are practically the same in each of the three rabbits, viz. I, 0·263, II, 0·275, III, 0·273.

When glycogen is added in addition to the fluoride, the inorganic phosphate falls to a very low level, the quantities being too small to permit of accurate determination.

Similar experiments were carried out on strychninised rabbits, and the results may be compared with those obtained in normal resting rabbits.

Rabbit IV. The strychnine was injected in increasing doses at 10-minute intervals for 45 minutes. The animal gradually became more spastic and 5 minutes after the last injection developed strong convulsions and died.

	% H_3PO_4
In *N*/10 sodium fluoride	0·263
In 5 % trichloroacetic acid	0·354
In 0·9 % sodium chloride, heated 2 hours 40°... ...	0·456
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·531

Rabbit V was injected with strychnine in small doses over a period of 3½ hours. Ten minutes after the last injection it developed strong convulsions and died in 5 minutes.

	% H_3PO_4
In *N*/10 sodium fluoride, after 6 hours 12°	0·443
In *N*/10 sodium fluoride, after 7 hours 12°	0·440
In 5 % trichloroacetic acid	0·360
In 0·9 % sodium chloride, heated 2 hours 40°... ...	0·466
In 2 % sodium bicarbonate, heated 2 hours 40° ...	0·478

In both animals the preformed or A value is about the normal, but the B value is lower than normal. This gives a lower lactacidogen value than normal, in one case 0·177 %, in the other 0·118 %.

The fluoride values are much higher than those found in resting rabbit's muscle. In the second rabbit the fluoride value was actually higher than the A value. It cannot be the inorganic phosphate that is lacking for synthesis, since this is as high as in the normal animals.

Cohn [1921] found for the lactacidogen values in the pale muscle of the strychninised rabbit figures ranging from 0·1286 % to 0·2057 %, with an average of 0·1635 %.

The values obtained in the above experiments may be summarised as follows:

	% H_3PO_4		
	Synthesis.		Hydrolysis.
	C ←	A	→ B
Resting muscle	0·044	0·356	0·576
,, ,,	0·057	0·332	0·601
,, ,,	0·100	0·373	0·623
Fatigued ,,	0·263	0·354	0·531
,, ,,	0·440	0·360	0·478

It is of interest to note that Embden and Adler [1921], using 10 g. of muscle, obtained for pale resting rabbit's muscle A values from 0·2008 % to 0·2755 % (average 0·2442 %) and B values from 0·5295 % to 0·6101 % (average 0·5712 %); and for lactacidogen values varying between 0·2800 % and 0·4093 % (average 0·3179 %). Using red muscle they obtained somewhat lower values.

Dog Muscle.

Effect of Stimulation.

Exp. 1. The dog was anaesthetised with ether. The sciatic nerve on one side was exposed, severed and the distal portion stimulated for 40 minutes with single induced shocks and then for 10 minutes with interrupted tetanising stimuli. The limb acted against a weight, and at the end the muscles were almost completely fatigued. The muscles in the resting and in the fatigued limbs were then exposed, sprayed with ethyl chloride and corresponding portions excised. They were placed in an ice-cold vessel and rapidly minced. Similar portions were at once placed in the three solutions and weighed.

	% H_3PO_4	
	Resting	Fatigued
In 5 % trichloroacetic acid	0·259	0·225
In 2 % sodium bicarbonate, heated 1 hour 40°	0·457	0·396
In *N*/10 sodium fluoride, 1 hour 12°	0·067	0·277

Both the A and B values of the muscle on the resting side are higher than those on the fatigued. On the resting side the B value is 76·4 % higher than the A. This corresponds to a store of lactacidogen which furnishes on complete breaking down this increment in free H_3PO_4. On the fatigued side the increment in free H_3PO_4 is practically the same although the absolute values (A and B) are down.

On the resting side the fluoride has caused a decrement in free H_3PO_4 amounting to 73·3 %. On the fatigued side the fluoride has, instead of producing a decrement in free H_3PO_4 from the A value, given rise to a 23·1 % increment. The stimulated muscle, which contains only 0·171 % of lactacidogen, is unable to synthesise in the presence of fluoride, whereas the resting muscle (0·198 % of lactacidogen) readily produces this effect.

Exp. 2. The dog was anaesthetised with ether, and one sciatic nerve was exposed and cut. A large proportion of the animal's blood was removed by perfusion with normal saline.

When the nerve was stimulated the muscles appeared to become fatigued very quickly—in about a minute. A specimen was taken into fluoride and the stimulation was continued for 5 minutes and again for 15 minutes.

	% H_3PO_4	
	Resting	Stimulated
In $N/10$ sodium fluoride	0·069	—
,, ,, ,, stimulated 1 minute	—	0·121
,, ,, ,, ,, 5 minutes	—	0·120
,, ,, ,, ,, 15 ,,	—	0·126
In 2 % sodium bicarbonate, heated 2 hours 40°	0·400	0·310

The action of the fluoride was again more marked on the resting than on the fatigued side. The total phosphate (preformed and lactacidogen) showed a marked diminution (22·5 %) compared with that obtained on the unstimulated side.

In this animal some experiments were devised to find out how treatment with sodium chloride affected the fluoride synthesis in the muscles on the resting side.

Resting muscle.	% H_3PO_4
In $N/10$ sodium fluoride, 1 hour 11°	0·069
In fluoride 1 hour, then washed and placed in saline 2 hours 40°	0·124
In saline 2 hours 40°	0·603
In saline 2 hours 40°, then left overnight in fluoride	0·361
In saline 20 minutes 40°	0·238
In saline 20 minutes 40°, then in fluoride for 1 hour	0·116

The maximal disintegration of the lactacidogen occurred in the specimen kept in 0·9 % sodium chloride at 40° for 2 hours.

After a preliminary 1 hour action of fluoride followed by 2 hours' action of chloride at 40°, where the fluoride building-up action is counteracted by the chloride breaking-down action, the free H_3PO_4 is brought approximately to the same level as by the fluoride alone acting upon stimulated muscle. When, on the other hand, the breaking-down action of chloride is first allowed to take place and then the muscle is treated with fluoride, the former effect is dominant, so that the fluoride is not able to synthesise to the same extent.

Exp. 3. The dog was anaesthetised with ether. The sciatic nerve on one side was severed and stimulated for 5 minutes with interrupted tetanising stimuli. The muscles showed no evidence of fatigue in this time. Specimens were quickly taken from each side into fluoride. Transfusion was then begun and most of the animal's blood was replaced by saline. At the same time stimulation of the sciatic nerve was continued. The muscles quickly became fatigued—in about 5 minutes fatigue was complete. Specimens of muscle from both sides were then taken.

	% H_3PO_4	
	Resting	Stimulated
Before transfusion:		
In $N/10$ sodium fluoride	0·081	0·077
After transfusion:		
In $N/10$ sodium fluoride	0·071	0·148
In 2 % sodium bicarbonate, heated 2 hours 40°	0·373	0·264

The resting muscle, after heating with bicarbonate, showed a higher H_3PO_4 value (by 41·3 %) than the fatigued.

The fluoride synthetic effect was not changed by 5 minutes' stimulation prior to transfusion, but after the onset of fatigue subsequent to transfusion the synthetic effect of the fluoride was markedly lowered.

A few experiments carried out on the resting muscle alone with regard to the effect of fluoride after the action of chloride at 40° for 2 hours showed no appreciable synthetic effect at 0°.

Exp. 4. A dog was anaesthetised with ether. The sciatic nerve was exposed on one side and stimulated for 20 minutes with interrupted tetanising stimuli. At the end of this period the muscles were still contracting vigorously. Perfusion was commenced and at the same time the animal was bled from the carotid. Stimulation of the sciatic nerve was continued. Unfortunately the heart failed in about two minutes and the animal died. The muscles were quickly excised and examined in the usual way.

	% H_3PO_4	
	Resting	Stimulated
In *N*/10 sodium fluoride	0·079	0·178
In 5 % trichloroacetic acid	0·244	0·219
In 2 % sodium bicarbonate, heated 2 hours 40°	0·375	0·368
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·392	0·374

Here again the H_3PO_4 value in the resting muscle treated with fluoride is much lower than that of the stimulated muscle treated in a similar way.

The A and B values are both lower in the stimulated muscle. The lactacidogen value is slightly higher in the stimulated muscle; but when acted upon by fluoride the free H_3PO_4 is diminished by 67·6 % in the resting muscle and only by 18·7 % in the stimulated.

CAT MUSCLE.

A cat was anaesthetised with ether. The vessels were perfused with saline and while the blood was gradually being washed out the sciatic nerve on one side was exposed and stimulated. After being tetanised for 10 minutes with short interruptions the muscles were completely fatigued.

	% H_3PO_4	
	Resting	Stimulated
In *N*/10 sodium fluoride	0·124	0·212
In 5 % trichloroacetic acid	0·315	0·278
In 0·9 % sodium chloride, heated 2 hours 40° ...	0·477	0·392
In 2 % sodium bicarbonate, heated 2 hours 40°	0·445	0·345

Both the A and B values of the fatigued muscle are lower than those of the resting, and the difference between the A and B values shows in the resting muscle an increment of 41·2 % in free H_3PO_4 and in the fatigued muscle an increment of 24·1 %. The absolute increments are 0·130 and 0·067 % respectively. The lactacidogen value of the stimulated muscle is, therefore, much below that of the resting.

The fluoride effects show a synthesis in both the resting and the fatigued, well marked in the former (0·191 %) and only slight in the latter (0·066 %).

Estimations of glycogen and in many cases of glucose and lactic acid were performed in these muscle specimens, with the object of correlating the carbohydrate changes with those of the phosphoric acid. In this preliminary communication, however, attention has been directed solely to the phosphate changes. It will be of interest to see if the addition of glycogen to the fluoride in the case of fatigued muscle can restore the synthesising power to the level normally found in unfatigued muscle.

Summary.

1. Glycogen when added to the fluoride acting on resting muscle causes practically a disappearance of free phosphate from the mixture.

2. During muscular fatigue there is a slight diminution of the total phosphate (preformed and lactacidogen) and also of free phosphate in the muscle.

3. In fatigued muscle, with practically as much preformed phosphate as in the resting, the synthetic action of fluoride is greatly diminished.

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XXXVII. THE PRODUCTION OF ACIDOSIS BY INGESTION OF MAGNESIUM CHLORIDE AND STRONTIUM CHLORIDE.

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(*Received March 2nd, 1925.*)

Haldane, Hill and Luck [1923] put down the acidosis caused by the ingestion of $CaCl_2$ to a precipitation of $CaCO_3$ in the intestine. If this is correct, similar effects are to be expected on taking $MgCl_2$ and $SrCl_2$. In the former case the reaction occurring in the gut would be:

$$MgCl_2 + 2NaHCO_3 = MgO_2H_2 + 2NaCl + 2CO_2,$$

in the latter:

$$SrCl_2 + 2NaHCO_3 = SrCO_3 + 2NaCl + CO_2 + H_2O,$$

the insoluble hydroxide or carbonate being excreted rectally, the NaCl reabsorbed. The net effect would be the substitution of $NaHCO_3$ by NaCl in the blood and tissues, just as if an equivalent of HCl had been taken. A certain amount of the bivalent metal would also be precipitated and excreted as soap, with the same effect on the reaction of the tissues.

On two occasions J. B. S. H. drank a litre of water in which 25 g. $MgCl_2$ were dissolved. The alveolar CO_2 pressure, estimated by the method of Haldane and Priestley [1905], fell from 39·3 to 32·8 mm. and from 37·2 to 31·9 mm. This fall was completed within 5 hours of taking the salt, but the pressure had not returned to normal on the subsequent mornings. The increased breathing might conceivably have been due to a stimulation of the respiratory centre by Mg; however, an analysis of the urine secreted during the 24 hours after taking the salt left no doubt of the presence of a true acidosis. While on four normal days the acid titrated to phenolphthalein varied between 57 and 61 millimols, 76 were excreted after taking $MgCl_2$. The ammonia, estimated by Malfatti's [1908] method, which had varied between 69 and 75 millimols, rose to 107. The salt caused intense diarrhoea, continuing for more than 24 hours. A sample of faeces was analysed qualitatively. The filtrate was alkaline to litmus and contained Mg and Cl. The residue, insoluble in water, was almost wholly soluble in dilute HCl. It gave no reactions for Ca or carbonate but contained much Mg. It therefore probably consisted largely of MgO_2H_2. An analysis kindly performed by Miss Watchorn showed that 0·514 g. of Mg as against about 0·14 g. on normal days had been excreted in the urine within 24 hours. The total was only 8 % of that ingested.

60 g. of $SrCl_2 . 6H_2O$ were drunk in a litre of water. The alveolar CO_2 pressure fell from 40·3 to 33·1 mm. and was still low next morning. The urine secreted within 24 hours after taking the salt contained 68 millimols of acid and 102 of NH_3. There was therefore an acidosis. Besides diarrhoea, a slight headache, and malaise lasting for several days were experienced. The filtrate from a sample of faeces was neutral to litmus and contained much chloride, but only traces of alkaline earths. The residue insoluble in water was partly soluble in dilute HCl with effervescence. The alkaline earth metals were precipitated as oxalate and converted to nitrate. Only a trace of this was soluble in amyl alcohol. The residue gave a brilliant Sr spectrum. The insoluble part of the faeces therefore contained $SrCO_3$ and perhaps strontium soaps, but very little Ca. The alkaline earths of the urine were precipitated as oxalates, ashed, converted to nitrate, separated by extracting the $Ca(NO_3)_2$ with amyl alcohol, and estimated volumetrically as oxalate. The day's urine contained 1·46 g. of Sr, or 7·4 % of the total taken, and 288 mg. of Ca, as against a normal of 127–178. The excess is fully accounted for by the acidosis [Stewart and Haldane, 1924] and there is no evidence of a displacement of Ca by Sr.

There was a possibility that any violent diarrhoea might cause an acidosis through loss of bicarbonate. So, as a control, 40 g. $Na_2SO_4 . 10H_2O$ were drunk in 800 cc. water. This produced copious diarrhoea of a p_H about 7·5, but no change in the alveolar CO_2 pressure.

SUMMARY.

Ingestion of $MgCl_2$ or $SrCl_2$ causes acidosis in man.

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XXXVIII. OXIDATIVE DEAMINATION BY A BASIDIOMYCETE ENZYME.

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(*Received March 2nd, 1925.*)

The oxidative deamination of amino acids by tyrosinase was first observed by Chodat and Schweizer [1913], who demonstrated qualitatively that an enzyme prepared from the potato, when in the presence of oxygen and at an alkaline reaction, oxidised certain amino acids with the production of carbon dioxide, ammonia, and an aldehyde containing one carbon atom less than the original amino acid. The above reaction is stated to proceed more readily and completely in the presence of certain phenols, Schweizer [1917] considering that this acceleration was caused by the combination of the phenol with the products of the reaction. Both *p*-cresol and catechol are mentioned in this connection, but in the experiments described by these authors *p*-cresol alone was used.

The fact that this phenol is readily oxidised by tyrosinase with the production of an orange-coloured substance was first observed by Chodat [Chodat and Staub, 1907], who subsequently found that if certain amino acids were added to *p*-cresol and tyrosinase, fluorescent cresol-blue pigments were formed; he did not, however, at first ascribe this phenomenon to the oxidation of the amino acids.

Chodat and Schweizer [1915] state that it is not known whether the oxidation of tyrosine proceeds in the same manner as that detailed for amino acids such as glycine and alanine; Schweizer [1917] describes, however, the detection of small quantities of ammonia with Nessler's reagent after tyrosine had been acted upon by the enzyme in the presence of *p*-cresol.

For the work described in this paper a tyrosinase was used from the basidiomycete *Lactarius vellereus*. A crude enzyme extract was prepared according to the method described by Onslow [1920] for the phanerogams. The plant tissue was rapidly and thoroughly pounded with 97 % alcohol and was repeatedly filtered to dryness on a Buchner funnel; the residue was then extracted with phosphate buffer of p_H 7·8 for about 10 minutes and filtered, the filtrate containing a crude solution of the enzymes present, including an exceedingly active tyrosinase. The extract gave a negative ninhydrin reaction, demonstrating its freedom from amino acids and polypeptides which might have participated in the reactions, described by Chodat and Staub, between phenols and these substances in the presence of tyrosinase. Small amounts only of the enzyme were made each time; the extracts, however, were found to retain their strength for some days if kept in a refrigerator.

The apparatus used by us to investigate the oxidation of amino acids was a modified form of the Barcroft differential manometer, four instruments being shaken mechanically side by side in a water bath at 16°, this temperature being preferred to a higher one partly because of the great rapidity of the initial oxidations, and partly to facilitate speedy equilibration rather than on account of considerations of the optimum temperature for the tyrosinase reaction. The strengths of the solutions of amino acids and phenols were those used by Chodat and Schweizer [1913], the solvent being in every case phosphate buffer of p_H 7·8.

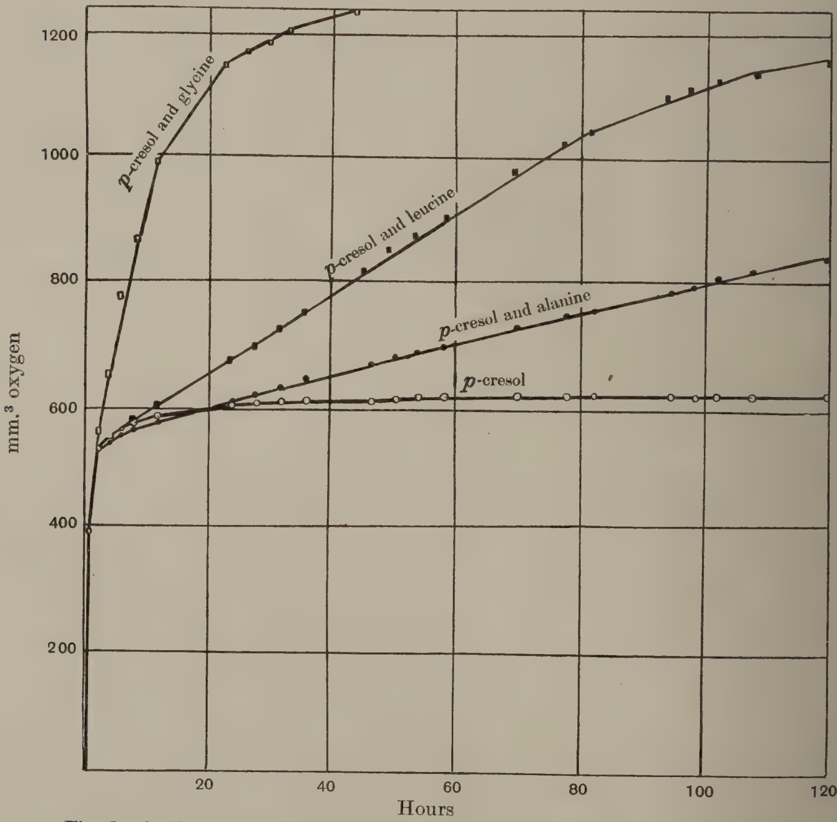

Fig. 1. Oxidation of glycine, leucine and alanine in the presence of *p*-cresol.
2 mg. *p*-cresol. 4·5 mg. glycine.
Equimolecular solutions of leucine and alanine

The enzyme extract alone was found to be entirely unable to oxidise glycine, alanine and leucine, though tyrosine showed a rapid oxygen uptake, accompanied by melanin formation. In the presence of *p*-cresol, however, the three former amino acids were readily oxidised (see Fig. 1). It will be seen that the oxygen uptake of glycine is more rapid than that of leucine, which, in its turn is oxidised more readily than is alanine. With the enzyme preparation used, oxidation of glycine was complete in about 40 hours, the amount of oxygen used in excess of that absorbed by *p*-cresol alone being approximately that theoretically required for the equation

$$CH_2(NH_2).COOH + O \longrightarrow HCHO + NH_3 + CO_2.$$

The oxidation of leucine or alanine was incomplete after 100 hours, when the shape of the curve for leucine indicates a diminution of the velocity of the reaction, possibly due to the inactivation of the enzyme. The form of these oxidation curves seems to indicate the existence of a coupled reaction, reminiscent of that obtained in the oxidation of unsaturated fats by the sulphydryl grouping. It is noteworthy that the concentration of *p*-cresol does not appear to be a limiting factor of the velocity of the reaction; further work on the kinetics of the reaction is now in progress.

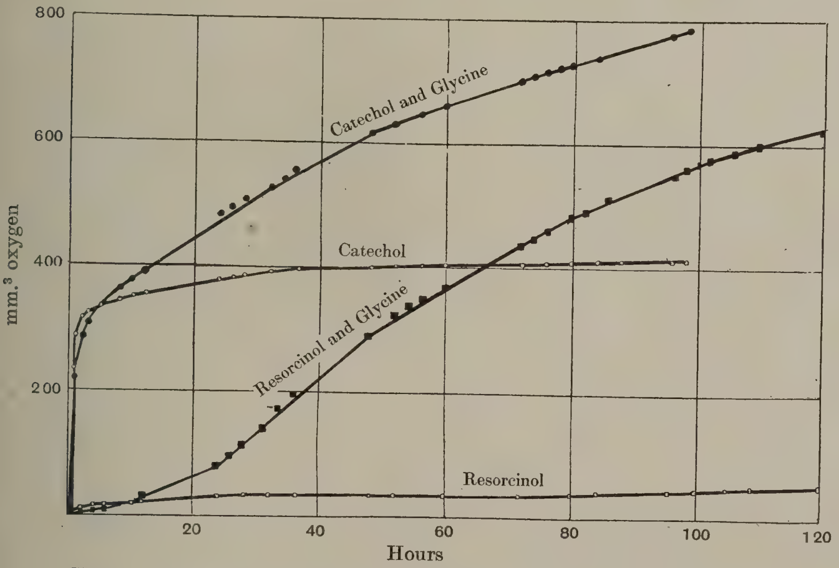

Fig. 2. Oxidation of glycine in the presence of (*a*) catechol, (*b*) resorcinol.

4·5 mg. glycine.	2 mg. catechol.
4·5 mg. glycine.	2 mg. resorcinol.

p-Cresol was then replaced by an equimolecular solution of catechol, and again oxidation of glycine was obtained. The initial oxygen uptake of catechol when catalysed by the *Lactarius* enzyme was extremely rapid, therefore a special form of bottle was used on the Barcroft manometer, a measured volume of the enzyme extract being introduced, from a side burette fitted into the bottle, after equilibrium had been attained; after addition of the enzyme the taps were immediately turned so as to connect the bottles and the manometer. The result of this experiment is shown in Fig. 2. It will be seen that the oxidation of glycine in the presence of catechol is less rapid than in that of *p*-cresol, when an enzyme extract of approximately similar strength is used, oxidation being incomplete in 100 hours. For these experiments Merck's resublimed catechol was used; it may be noted that one molecule of catechol upon oxidation takes up one molecule of oxygen.

Using the same technique, one of us (M. E. R.) has shown that glycine and leucine are oxidised in the presence of *p*-cresol and a potato oxidase, the method of preparation of the enzyme being the same as that described for *Lactarius*. Glycine is also oxidised in the presence of catechol. The curves obtained are similar to those given for the oxidations in the case of *Lactarius*.

p-Cresol and catechol were then replaced by resorcinol. The reaction in the presence of this phenol is somewhat different, oxidation taking place only after a latent period which sometimes extended to 15 hours, the phenol itself without the amino acid in the presence of the enzyme taking up practically no oxygen during this period. Resorcinol, unlike catechol and *p*-cresol, is not autoxidised if allowed to stand exposed to the air for a considerable period, manifesting at the end of this time no peroxide formation on testing with guaiacum and horse-radish peroxidase.

The enzyme extract was found to be unable to oxidise glycine in the presence of carefully buffered solutions of *p*-hydroxybenzoic acid or aldehyde, nor were these substances themselves oxidised. Similarly the oxidation of protocatechuic acid and aldehyde was extremely slow in comparison with that of catechol, though there was evidence of oxidative deamination of glycine in the presence of the former.

Tyrosine itself is unable to replace *p*-cresol in the oxidation of amino acids. When glycine was added to a saturated solution of tyrosine in phosphate buffer of p_H 7·8, the oxygen uptake was no greater than that of a similar solution of tyrosine in the absence of glycine; on the other hand, no inhibition of the speed of oxygen uptake of tyrosine when in the presence of other amino acids was observed.

The decrease of amino nitrogen at the close of the reaction was investigated by means of the micro form of Van Slyke's apparatus. The solutions were removed from the bottles of the Barcroft manometer by means of a pipette, and the amino nitrogen content was compared with that of a control solution which had been prepared with boiled enzyme and which had been shaken concurrently in another Barcroft apparatus.

Results obtained were as follows:

Substance	O_2 uptake of amino acid at 16° mm.^{3}	Amino N of control mg.	Amino N after deamination mg.	Loss of amino N mg.	Theoretical loss calc. from O_2 uptake mg.
Glycine + *p*-cresol	640	0·844	0·054	0·790	0·800
Leucine + *p*-cresol	540	0·875	0·265	0·610	0·628
Glycine + catechol	380	0·842	0·346	0·496	0·509

A decrease of amino nitrogen, and the production of ammonia, was observed also in the case of glycine and resorcinol in the presence of the enzyme.

The results of estimations of the ammonia produced during the course of the reaction did not correspond exactly with the observed loss of amino nitrogen, giving slightly lower values. Estimations of the total nitrogen content, by Kjeldahl's method, before and after oxidation had taken place, showed however that no loss of nitrogen as ammonia from the alkaline fluid had occurred. It is possible that the aberrant nitrogen may have taken part in the production of the coloured complex.

That Chodat and Schweizer observed oxidation of amino acids by tyrosinase alone, though acceleration of the reaction was obtained in the presence of certain phenols, may possibly be explained by the supposition that the enzyme extract used by them, prepared from the potato, may have contained traces of an autoxidised *ortho*-dihydroxy compound [Onslow, 1920]. Schweizer states that he prepared tyrosinase according to the instructions given by Chodat in Abderhalden's *Handb. d. biochem. Arbeitsmethoden.* The preliminary stages of this method include mincing of the potatoes and maceration with water of the crude alcoholic precipitate containing the enzyme, during which processes a certain amount of oxidation is difficult to avoid. Autoxidised substances might possibly be adsorbed on the proteins present together with the enzyme, and be unremoved by subsequent reprecipitations.

The basidiomycete *Lactarius vellereus* does not appear to contain *o*-dihydroxy substances [Robinson, 1924]; the enzyme extracts used in our experiments, moreover, did not undergo spontaneous darkening or oxidation on being shaken in air for a considerable period.

SUMMARY.

A crude enzyme extract, containing tyrosinase, from the basidiomycete *Lactarius vellereus* is unable to oxidise the amino acids, glycine, leucine and alanine unless certain phenols be present. Oxidative deamination of glycine has been brought about in the presence of *p*-cresol, catechol and resorcinol. *p*-Cresol cannot be replaced in this reaction by *p*-hydroxybenzoic acid or aldehyde.

A possible explanation for the divergence of our results from those of Chodat and his co-workers is given in the text.

In conclusion we wish to express our thanks to Sir F. G. Hopkins, F.R.S., and to the Hon. Mrs Onslow for their continual interest during this work and for much valuable advice.

Note [by R. A. McCance]. Although neither *p*-cresol nor resorcinol decolorises methylene blue either alone or in the presence of tyrosinase, the system, glycine (or any amino acid) plus *p*-cresol, resorcinol, etc., plus tyrosinase has been found to decolorise methylene blue freely. Of the amino acids tried, glycine is the most active in this connection. Tyrosine can act in this connection in its capacity as an amino acid.

A study of this reaction has been made with a number of substances and the results and conclusions as to the nature of the chemical changes involved will be published shortly.

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XXXIX. THE RELATION OF PECTOSE AND PECTIN IN APPLE TISSUE.

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(*Received March 5th, 1925.*)

It has long been maintained that pectose, or proto-pectin as it is called by some authors, is an insoluble constituent of plant tissues, associated with cellulose in the cell-walls but exhibiting none of the properties of that substance. It has also been claimed by the majority of investigators that during the ripening of fruit, pectose decomposes into a soluble form, generally known as pectin, which consequently appears in increasing amounts in the expressed cell-sap as the fruit matures. In the unripe fruit, however, pectin occurs in negligible quantities only.

Concurrently with this disturbance in the pectic constituents, the fruit exhibits signs of softening as ripening proceeds. Microscopic examination of the tissues shows that this softening is associated with structural relations and staining and chemical reactions of the cell-wall which can only be interpreted as due to the conversion of an insoluble substance like pectose into soluble pectin.

In advanced stages of over-ripeness and during senescence the pectic changes become very much pronounced. In this condition the tissues are readily disintegrated, the cell-walls become markedly thinner, and the cells ultimately separate from one another owing to solution of their middle lamella substance.

It has been found possible to locate the pectic substances in the cell-walls by staining with ruthenium red, and to follow their distribution throughout the development of the fruit[1]. Pectic substances have been located in the cell-walls and middle lamella, and periodic examinations of the apple tissue as the season advances confirm the view that the physical modifications in mature fruit are due to pectic changes, and that the processes of ripening and physiological breakdown are intimately associated with the progressive change of an insoluble pectic substance into a soluble modification. As a result of chemical work already published in part [Carré, 1922] and the unpublished microscopical studies referred to above, it would seem difficult to hold any other view than that pectose (proto-pectin[1]) is chemically different from the pectin to which it gives rise, both these substances occurring in the tissues of ripe fruit.

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[1] This work has been carried out in conjunction with Dr A. S. Horne and will shortly be ready for publication.

An exactly opposite view, however, has been put forward by F. Tutin [1923], who vigorously disputes the conception of pectose and pectin as chemically different substances in the tissues, and upholds an alternative explanation of the phenomena to be observed during the ripening processes of fruits.

Tutin regards the existence of pectose as "purely hypothetical," and considers that the pectic material of the tissues consists of pectin only, which is "held in its typical soluble form throughout the development of the fruit." He maintains that ripening is in no way associated with chemical changes in the pectic components of the tissues, but that the apparently insoluble condition of the pectin (especially in hard unripe fruit), which is usually explained by the existence of pectose, is really due "to the great difficulty in attaining by mechanical means, really efficient disintegration of the hard unripe tissue."

Tutin therefore suggests that extraction with water alone should be sufficient to dissolve out the whole of the pectin thus held in the tissue, provided that complete disintegration of the material can be obtained. He consequently regards the ease with which pectin is extracted from ripe fruit as compared with unripe to be solely due to alterations in the physical condition of the fruit, and in no way connected with any chemical change in the pectic constituents themselves. Tutin also suggests that apart from the difficulties of mechanical disintegration, another factor is at work, namely, the action of certain constituents of the tissues, which tends to retain the pectin and prevent its ready removal with water. In support of this, he states that solutions of pectin approximating to a concentration of 0·5 % cannot be filtered without undergoing change in concentration, owing to retention of some of the dissolved pectin by the filter paper. He suggests that when attempts are made to extract the pectin from the tissues by treatment with water, the cell walls retain pectin in a similar manner.

It would seem impossible, however, to accept such a view. The question of the retention of pectin during filtration through various media has been investigated by the author, and it has been found that the amount absorbed varies directly with the concentration of the solution employed, but that solutions containing less than 0·9 to 1·0 % of pure pectin exhibit no alterations when filtered through ordinary filter paper. If on the other hand the solution to be filtered has a concentration greater than 1·0 % of pectin, an appreciable amount may be taken up. Such concentrations of pectin are however greater than that obtained in normal juice or under the experimental conditions of the usual extraction methods.

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[1] As the word "pectose" receives the support of the majority of workers in this field, it is here adopted in preference to "proto-pectin."

These results are illustrated by the following examples:

Effect of Filtration on Pectin Solutions. Concentration of Pectin Solution (100 *cc.*).

Before filtration		After filtration	
	%		%
1	0·025	1	0·025
2	0·115	2	0·116
3	0·227	3	0·226
4	0·760	4	0·759
5	0·870	5	0·873
6	0·170	6	0·148

Experimental evidence has also been obtained showing that pectin solutions of concentrations similar to the above are unchanged by passage through a dense mass of tissue from which pectin had been previously removed by water. Since the methods of extraction of pectic substances involve the use of a large bulk of water, the concentrations of pectin rarely exceeding 0·05 %, such solutions will be unchanged by filtration through paper or in their passage through the tissues themselves. It is therefore evident that the holding back of pectin cannot be regarded as responsible for the supposed retention of the pectic constituents of the unripe tissues.

Tutin describes two further experiments which he considers afford conclusive evidence for his views.

In an earlier communication, Carré [1922] showed that pectose and pectin could be removed separately from apple tissues by first extracting the soluble pectin with *cold* water, and subsequently treating the residue with dilute hydrochloric acid, thus converting pectose into pectin which can then be removed with cold water as before. Tutin has carried out a similar experiment but interprets it very differently. He treated three equal quantities of apple residue obtained during the production of cider. One sample was exhaustively extracted with boiling water in a Soxhlet apparatus, and 1·4 g. pectin was thus obtained. A second sample was autoclaved at 110° with dilute acid for 1 hour and subsequently extracted with boiling water as before. The extract yielded 1·6 g. pectin.

Tutin assumes that if both pectin and pectose had originally been present in the tissues, the water extraction would have yielded soluble pectin only, whereas the subsequent acid treatment should have produced a much larger amount, due to the decomposition of pectose. Since the weight of pectin in both cases was about the same, Tutin concludes that no pectose can have been present in the samples.

In criticism of these deductions the following observations may be made: (1) The material used for the experiment was the "pomace" or dry press-cake from the cider press. It is therefore obvious that the juice expressed from the apple during the production of the cider would have carried away with it the soluble pectin originally present in the fruit. Hence Tutin's experiment represents nothing more than two different methods of extraction of the insoluble pectose remaining in the residue after the removal of the soluble pectin. (2) It has been proved by the present writer that *cold water only* can be used to extract the soluble pectin from tissues, since extraction with hot water induces slow conversion of the pectose into pectin, presumably by a process of hydrolysis analogous to that effected by dilute acid. It follows therefore that the 1·4 g. of pectin obtained by Tutin with boiling water actually represents a portion at least of the pectose present in the original sample. Traces of acid remaining in the "pomace" would also tend to accelerate the decomposition of the pectose. (3) The method of extracting pectin by autoclaving with hydrochloric acid at 110° has been found invariably to give unsatisfactory results, probably owing to the fact that such treatment tends to bring about the decomposition of the pectin thus produced. The method finally adopted for the quantitative extraction of pectose depends upon boiling the material with $M/100$ hydrochloric acid and this process is found to give reliable results.

The following experiment demonstrates the accuracy of this method as compared with the autoclave method employed by Tutin. Ten uniform samples of apple pulp treated with $M/100$ HCl at 100° gave a mean pectose yield of 0·76 %, while ten similar samples of material extracted by the autoclave method gave a mean of 0·67 % pectose, showing that the results obtained by the latter method tend to be too low.

This indicates that the 1·6 g. of pectin obtained by Tutin from his second sample using the autoclave method is also too low a result and does not represent the actual pectose content of the sample. It would also seem that the evidence derived from this experiment is insufficient to justify his assertion of the non-existence of pectose.

In his second experiment Tutin describes successive extractions carried out in four stages of pectin from unripe apple tissue. (I) A weighed quantity of dried material previously soaked in alcohol was pounded in a mortar and soaked for twenty-five successive periods of 24 hours each, the volume of water used for each extraction being 1300 cc. The resulting solutions of pectin were evaporated under diminished pressure and estimated. The pectin content of each of the last ten extracts was small and apparently constant. Tutin considers this slowing down in the removal of the pectin to be due to the resistance offered by the undisintegrated portions of the material. (II) The material was next dried and thoroughly ground with sand in order to increase disintegration and the residue was extracted as before till only negligible quantities were obtained. (III) The process of grinding with sand and extracting with water was repeated. The residue examined microscopically showed that disintegration had been very thorough, although groups of cells were observed to have escaped the grinding process. Tutin therefore concludes that a small amount of pectin necessarily remains in the residue. He suggests that complete disintegration "would be most readily accomplished by resorting to the treatment with dilute hydrochloric acid employed by Carré for the hydrolysis of the supposed protopectin[1]" (pectose). (IV) Tutin therefore treated the residue with $M/20$ HCl in an autoclave at 110° and the resulting solution contained very little pectin. It is probable that the yield is too low in consequence of the method of procedure. Subsequent treatment with hydrochloric acid by this method produced no further yield of pectin.

The whole pectic constituents of the tissues having been removed with water alone, Tutin therefore concludes that water-soluble pectin is the sole substance of pectic nature in the tissues, and that the problem of estimating the pectin content of fruits depends mainly on complete disintegration of the tissues in order to facilitate its extraction by water.

The explanation put forward by Tutin is the one which suggested itself to the writer in the earlier stages of pectin work, but it has been found totally inadequate to interpret the various facts observed during the course of a more exhaustive research on the pectic constituents of apples. Experiments similar in procedure to those described by Tutin but of a more searching nature had already been devised before the publication of his results. These experiments afford conclusive evidence for the existence of pectose and pectin as definite chemical entities, and suggest that although mechanical disintegration is an important factor in the extraction of pectin, the whole problem cannot be reduced to such simple terms.

In criticism of Tutin's deductions it may be suggested that pectin is not originally present as such in the tissues as he supposes, but arises from pectose by a process of hydrolysis, the amount of pectin produced by any method of extraction depending on the efficacy of the hydrolytic agent employed. Hence treatment of the tissues with cold water, as Tutin describes in the foregoing experiment, will convert pectose into pectin by an extremely slow process of hydrolysis. This would account for the long period of time, approximately 40 days, which he found necessary to extract by means of cold water the whole of the pectin from his sample of apple.

Tutin accounts for the increased production of pectin as a result of grinding the material by assuming that increased disintegration favours the dissolving out of the soluble pectin, but it must be pointed out that grinding will also promote increase of hydrolysis and therefore increased yields of pectin by bringing fresh supplies of pectose in closer contact with the water. When the tissues are subjected to a more vigorous process of hydrolysis—for instance by treatment with water at 45°, or by the use of dilute acids or alkalis—the production of pectin is found to increase according to the efficacy of the agent employed. The accompanying table illustrates this point. Approximately similar yields of pectin were obtained from each, but the time required for the operation varied markedly with the hydrolytic power of the reagent.

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[1] It is difficult to understand why weak hydrochloric acid should be assumed to possess a disintegrating action on the tissues apart from chemical action. As a matter of fact such acid does not produce complete disintegration, but mere separation of the cells (*vide*, p. 264).

Method	Yield of pectin g.	Time required for operation hours
Cold water	0·80	600
Water at 100°	0·795	96
$M/100$ HCl at 45°	0·81	24
$M/100$ HCl at 100°	0·80	15

The following experiments (A and B) may also be quoted in support of the hydrolysis theory outlined above.

Details of Experiment A (Fig. 1).

100 g. of apple tissue was pressed in a hand press and washed with cold water to remove any soluble pectin initially present in the tissues. The total weight of pectin thus obtained in the three fractions amounted to 0·0400 g., the final fraction containing only a minute trace of pectin (0·0005). The volume of water used for the extractions on this and subsequent days was maintained at 500 cc. in order that the pectic content of successive fractions might be properly compared. The residue was then soaked in water for 24 hours and the pressing and washing repeated. It will be seen that this treatment produced an amount of pectin (0·0415 g.) as large as the yield on the previous day, although no mechanical disintegration had been resorted to. This marked development of pectin may be attributed to hydrolysis of pectose in consequence of: (*a*) the prolonged contact of the tissues with water, (*b*) the action of enzymes liberated from the cells crushed during the pressing operations, (*c*) the accelerating effect of any acid left in the tissues, and probably to a combination of all these factors.

Further extractions of the material (3rd–6th day) produced decreasing yields of pectin, the amount obtained on the 6th day being negligible. Grinding the residue with sand (7th and 8th day) resulted in a further small amount, probably in consequence of the exposure of fresh quantities of pectose to the hydrolytic action of the water.

If the extraction of the pectin was simply a matter of solution of the occluded material by the water, the grinding operations should have produced a far greater effect than was actually observed to be the case, since subsequent operations showed that approximately two-thirds of the total pectic material still remained in the tissue. This method of procedure was discontinued on the 8th day. A point was not reached at which no further amounts of pectin were produced, since hydrolysis with cold water, although extremely slow, is a continuous process. Microscopic examination of the residue at this stage showed that the tissues were almost entirely disintegrated, but staining with ruthenium red revealed substances of pectic nature abundantly present in the fragments of cell-walls and in the middle lamella regions. In order to hasten hydrolysis the residue was re-ground with sand (8th day) and boiled with water for 5 minutes. The amount of pectin then produced (0·0900 g.) was twice as great as that obtained on the first or second day of the experiment, and nine times as much as the amount produced by cold water alone in the preceding fraction.

The boiling was repeated daily till only small traces of pectin were extracted, the final fractions being approximately constant in amount. It will be seen from the table that the total weight of pectin obtained by boiling the residue with water is 0·4515 g., nearly three times the amount produced by cold water treatment.

It might be suggested on Tutin's theory that the large production of pectin with hot water is due to its greater solubility at the higher temperature, but experimental evidence indicates that the solubility of pectin is not appreciably greater in hot than in cold water. Neither can the production of pectin be due to enzyme action in this case since the temperature of extraction is destructive to enzymes. It seems probable therefore that the enormous and rapid development of pectin can only be attributed to the increased hydrolytic action of the water on the pectose at the higher temperature.

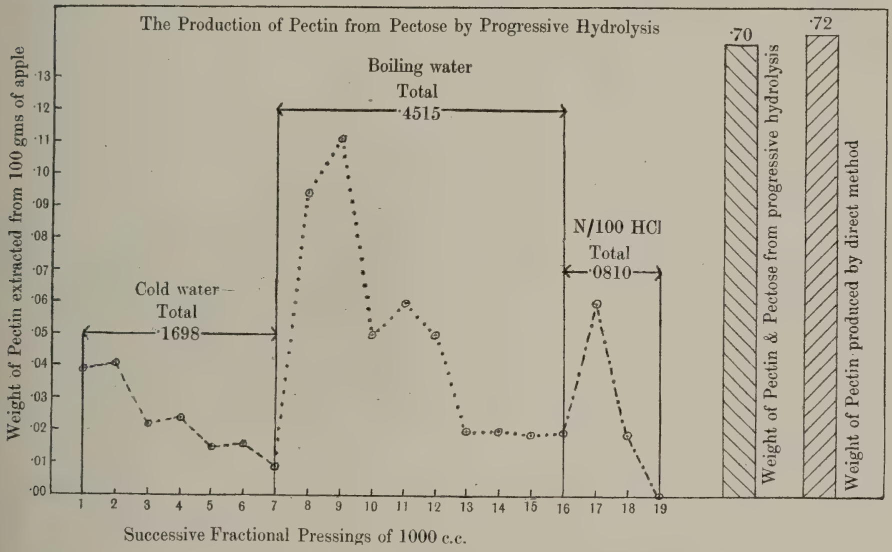

Fig. 1.

This conclusion is borne out by the further marked increase which was produced when the residue was treated (18th–20th day) with a more powerful hydrolytic agent, *i.e.* boiling dilute hydrochloric acid ($M/100$) for 15 minutes. After two such treatments no further trace of pectin could be obtained, and microscopic examination of the residue treated with ruthenium red showed that all the pectin constituents of the tissues had been removed. This effect of the acid in removing the pectin cannot be attributed to solvent action since pectin is only very slightly soluble in dilute acids. The above experiment suggests that if dilute hydrochloric acid be used for the hydrolysis of the pectose from the outset, the extraction should be effected more rapidly than with water alone.

EXPERIMENT B.

A second experiment was therefore carried out on a 100 g. sample of apple, similar to that used in Exp. A. The pulp was extracted with $M/100$ HCl at 100° for successive 3-hour periods. After each treatment the residue was pressed and washed to remove the bulk of the pectin produced. It was found that after three such extractions no further pectin was obtained. The total extracted was 0·72 g., which agrees very closely with the 0·70 g. obtained from similar material in Exp. A, allowing for the experimental error necessarily involved in the latter (see Fig. 1).

Experiment B.

100 g. apple tissue. Material extracted with $M/100$ HCl at 100°.

Extraction	g. of pectin obtained
1	0·4810
2	0·1943
3	0·0448
4	0·0000
Total pectin ...	0·7201

Microscopic examination of the residue showed that all the pectic material had disappeared, although the tissues were not so markedly disintegrated by the acid as by the grinding with sand in the former experiment. It was found that the cells were separate from one another, in consequence of the solution of their middle lamellae, but the cell-walls themselves were almost invariably unbroken, and preserved their original outline.

Microscopic investigations of the pectic substances have been carried out by the author in conjunction with Dr A. S. Horne, and the results, which will be briefly alluded to, confirm the opinions arrived at from the chemical side. The decomposition of the pectic substances has been followed under the microscope by treating in various ways sections stained with ruthenium red, which colours the pectic constituents. Irrigation of these sections with water produced considerable swelling of the stained areas followed by their very slow disappearance. This process was observed to be much more rapid with hot water, and very much accelerated when either dilute acid or alkali was employed. Solution of the middle lamella and ultimate separation of the cells were attained, but complete removal of the pectic components of the cell-walls involved no fundamental alteration in the appearance of these walls.

It was also found that if sections were irrigated with dilute ammonium oxalate solution, which has long been recognised as a solvent for pectic substances, the solution of the stained areas was very rapidly effected. The cells were again observed to separate from one another, but the cell-walls themselves remained entirely unbroken.

It is therefore apparent from the foregoing discussion of results obtained by the author, that Tutin's theory of the non-existence of protopectin, or pectose, cannot be supported by experimental evidence. It may be concluded, on the other hand, that pectose does actually exist as a practically insoluble constituent of the cell-walls of apple tissue, and that it can be converted into the soluble modification, known as pectin, by the action of water and by dilute acids or alkalis, the nature of the change being probably hydrolytic.

It is also evident that although mechanical disintegration is important in facilitating the process of hydrolysis, it is not, as Tutin claims, the essential factor in the removal of the pectic constituents of apple tissues, the necessity for disintegration being obviated if a sufficiently powerful reagent is used for extraction purposes.

SUMMARY.

It is shown by the author that Tutin's views on the non-existence of protopectin (pectose) cannot be maintained by the evidence afforded by his experiments. On the contrary, chemical investigations, borne out by microscopical work, indicate the presence of an insoluble pectic constituent in apple tissue, which is referred to as pectose, and which is converted by a process of hydrolysis into the soluble modification known as pectin.

This investigation has been carried out for the Food Investigation Board of the Department of Scientific and Industrial Research. The author wishes to express her thanks to Prof. V. H. Blackman and Dr D. Haynes of this department for their advice and criticism.

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XL. STUDIES ON THE METABOLISM OF ARGININE AND HISTIDINE.

PART I. ARGININE AND HISTIDINE AS PRECURSORS OF PURINES.

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(*Received November 17th, 1924.*)

The experiments described in this paper were undertaken in the hope of confirming the suggestion, advanced by Ackroyd and Hopkins [1916], that arginine and histidine are utilised in the animal body as precursors of purines. Not only did Abderhalden and his collaborators [1909, 1910], in testing this very idea, fail to obtain evidence in support of it, but since 1916 the validity of Ackroyd and Hopkins' conclusions has been called in question by several workers. Thus Lewis and Doisy [1918], maintaining normal men on purine-free high-protein diets of high and low content of arginine and histidine, found that no differences in the excretion of purines followed the ingestion of the two types of diet. More recently, Christman and Lewis [1923] have observed a marked diminution in the daily elimination of allantoin following the feeding of amino acids (glycine, alanine and glutaminic acid) to rabbits—results in agreement with those of Abderhalden and Einbeck [1909], who found the excretion of allantoin decreased when histidine was fed to dogs. On the other hand, Harding and Young [1919], feeding placenta (which is rich in arginine and histidine) to young dogs, with a control diet containing an equivalent amount of muscle protein (which is poor in these amino acids) found that the uric acid and allantoin excretions were parallel, and were markedly increased during the placenta feeding.

Not only does there remain some doubt as to whether arginine and histidine actually do function as purine precursors, but the whole of the evidence, both for and against, seems to have been derived from feeding experiments. In these circumstances, the author considered that some useful results might be obtained by employing the method of liver perfusion, in which the use of the single organ eliminates certain of the complications inherent in the use of the intact animal.

It is not necessary, of course, that a substance should be metabolised along one line only, and indeed, we have abundant examples to the contrary. Hence other suggestions as to the fate of arginine and histidine need not be considered contradictory to the hypothesis that they are or may be utilised in the synthesis of purines. In the case of arginine, Thompson [1917] has shown that hydrolysis to urea and ornithine takes place, and Felix [1923] claims that when arginine is perfused through the surviving liver, this hydrolysis is nearly quantitative. Dakin and Wakeman [1911] have demonstrated the production of acetoacetic acid; and Inouya [1912], Thompson [1917], and Gross and Steenbock [1921] have adduced evidence in support of the view that arginine is a precursor of creatinine. As to the changes undergone by histidine, there seems to be no suggestion other than that of Ackroyd and Hopkins [1916]. Its capability of conversion to acetoacetic acid [Dakin and Wakeman, 1911] and to glucose in the diabetic organism [Dakin, 1913] have been examined, but in both cases with negative results.

EXPERIMENTAL.

The perfusions were carried out with excised cat livers in the apparatus designed by Dixon [1922], Ringer's solution being used as the perfusion fluid.

The Control. One great objection to the perfusion method is the difficulty of obtaining an adequate control. If, as is usual, separate livers are used for the positive and control experiments, the comparison can only be valid when the number of perfusions is so large that the statistical method may fairly be applied to the results. The labour involved in so large a number of perfusions is necessarily very great, and an attempt has been made in the experiments here described to secure an efficient control and yet obtain a definite result with a relatively small number of perfusions.

Each liver was perfused for a definite time with a known volume of Ringer's solution only. This fluid was then removed and replaced by an equal volume of Ringer's solution to which had been added the substance under examination, the p_H and osmotic pressure being adjusted to the normal value. This was perfused for the same length of time with, of course, the same rate of flow and the same means of aeration—a slow stream of oxygen. Estimations were carried out on both perfusates. It remained to be shown that this procedure provided an *efficient* control—*i.e.* that any increase of the substance estimated was really due to the substance perfused. A series of experiments was therefore carried out in which the liver was perfused as before with two successive equal volumes of Ringer's solution to neither of which, however, was added any other substance. Estimations were carried out as before, and it was found that there was no increase in the second perfusate—in fact the second usually gave a slightly lower result than the first. This was true not only of allantoin, but also of urea, acetone bodies and sugar. It may fairly be considered, therefore, that this method of double perfusion affords an adequate control, each positive perfusion being compared with the corresponding blank. In this way it is possible to obtain a definite and reliable result with only some half-dozen perfusions.

The Criterion. The allantoin production was taken as the criterion of purine formation since allantoin has been amply shown to be the normal end-product of purine metabolism in the lower animals [*e.g.* Ackroyd, 1910, 1911, 1914]. It would seem that any increase in the end-product would be accompanied by an increase in the amount of each intermediate, so that in the case of purines, the more easily estimated uric acid might be used as the test-substance. Unfortunately, however, the colorimetric methods of estimating uric acid, which are best adapted for dealing with the small amounts here present, are not applicable in the presence of certain iminazole compounds which themselves give a blue colour with the various uric acid reagents.

Method of Estimation. The method of estimation was that of Wiechowski [1913] modified at the end to remove small quantities of iminazole compounds which escape precipitation in the earlier stages and are then thrown down by the allantoin reagent. Allantoin condenses with xanthydrol [Fosse and Heuille, 1923], while histidine does not, at any rate under the same conditions. The amount of condensation product isolated never corresponded to the whole of the allantoin, but, under strictly-defined conditions, it was always possible to isolate a fixed proportion.

A weighed amount of allantoin, mixed with histidine, was dissolved in the minimum amount of water and an equal volume of glacial acetic acid was added to the cold solution. Xanthydrol, in amount equal to twice the weight of the mixture (sufficient if the "mixture" was really pure allantoin), was dissolved in a volume of glacial acetic acid equal to that added to the allantoin solution; the two cold solutions were mixed, and allowed to stand for half an hour. Less time gave a smaller yield, more gave an impure product. The condensation product was then filtered off, washed four times with 5 cc. of cold water, dried at 100°, and weighed. As shown in the table below, this product was pure and corresponded to 90 % of the allantoin.

Weight of allantoin	·100 g.	·100 g.	·107 g.	·361 g.
Weight of histidine	—	·220 ,,	·250 ,,	·342 ,,
Weight of condensation product	·214 ,,	·214 ,,	·231 ,,	·777 ,,
Melting point of product (pure product melts at 214°) ...	214°	215°	213°	214°
% of allantoin recovered ...	89·9	89·9	90·0	89·8

In applying this method, the liquid, after perfusion, was made faintly acid with acetic acid, boiled and filtered from the precipitated protein. A measured volume of the filtrate was treated exactly as described by Wiechowski [1913]. After the addition of the allantoin reagent the liquid was allowed to stand overnight. The precipitate was then filtered off, suspended in hot water, and decomposed with hydrogen sulphide. The liquid was evaporated to dryness on the water-bath and the residue was thoroughly extracted with hot water. The combined extracts were evaporated to dryness and the residue was weighed. It was then dissolved in water and the solution evaporated to about 0·5 cc. An equal volume of glacial acetic acid was added and, after cooling, an equal volume of glacial acetic acid containing xanthydrol in amount equal to twice the weight of the residue. The condensation product of allantoin and xanthydrol was prepared as already described, its purity being tested by determination of the melting point. Throughout the estimation equal volumes of control and positive fluids were used and the liquids received exactly similar treatment. Thus the final weighings, without any calculation, gave a strict comparison of the amounts of allantoin in the two fluids, though, of course, not the absolute amounts.

Results and Discussion.

The results of one typical perfusion are given.

	Control	Positive
Time of perfusion	2 hours	2 hours
Volume of liquid	500 cc.	500 cc.
Volume taken for estimation ...	450 ,,	450 ,,
Weight of condensation product	37·0 mg.	37·6 mg.
Melting point	214°	214°

The perfusion of arginine or histidine through the excised liver caused no real increase in the production of allantoin. The slight increase shown in the experiment quoted is within the limits of experimental error. The results are therefore disappointing in that they fail to confirm the hypothesis that these amino acids are precursors of purines. They are, however, not sufficient of themselves to invalidate the hypothesis.

Summary.

The method of liver perfusion has failed to afford evidence in support of the hypothesis that arginine and histidine function in the animal body as purine precursors.

A method of controlling liver perfusion experiments is described.

A method is given for the estimation of allantoin in presence of iminazole compounds.

The author's thanks are due to Prof. G. Barger, F.R.S., for the interest he has taken in this work. The arginine was purchased with a grant from the Moray Fund of this University, hereby gratefully acknowledged.

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XLI. A METHOD OF MICROTITRATION.

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(*Received February 27th, 1925.*)

During a research to be published later it became necessary to obtain a continuous series of blood ammonia determinations on myself or other human subjects, but this object presented the serious difficulty that, according to the methods at present in use, about 10 cc. of blood would be required for each determination. I therefore attempted to devise a method in which only 1 cc. of blood should be required. One of the factors which make the determination of blood ammonia so difficult is that the quantity is extremely small.

The amount of ammonia in 100 cc. of blood is supposed to lie between 0·1 and 0·2 mg., which means that 1 cc. of blood can yield only 0·001 or 0·002 mg. NH_3 or about 0·1 $mm.^3$ of a normal solution.

It would be quite impossible to titrate this amount by any ordinary method, but, on consideration, I found that it would not only be possible to titrate the ammonia from 1 cc. of blood, but that by a suitably arranged microtitration it could be done with far greater accuracy than the amount from 10 cc. is titrated by a macrotitration.

As I think that the method of microtitration arrived at will make all titrations of small quantities much more accurate than is possible with ordinary macrotitrations, the method and the theory upon which it is based are published separately here.

The object of a titration is to measure how much of a solution of known concentration one must add to a certain volume of another solution of unknown concentration in order to bring a certain reaction between the two fluids to completion.

This involves two subjective estimations by the person who titrates: first to judge that the reaction is completed, secondly to measure the fluid added. That the reaction is completed is judged by means of an indicator[1]; the measuring is done by means of a burette.

The errors involved in a titration are, as in other cases, partly accidental, partly systematic. The accidental errors are mainly two: first, a misjudging of the colour of the indicator, so that the p_H wanted as end point is not reached or is passed—usually one may expect the actual end point to lie within 0·3 p_H-unit on each side of the p_H wanted. The second accidental error is due to the reading of the burette and depends mainly on the construction of this piece of apparatus.

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[1] In the following I shall speak only of acidimetric and alkalimetric titrations, though the reasoning will hold also for the other forms. For a complete discussion of the theory of these titrations see Bjerrum [1914].

The systematic errors depend on the fact that free acid and alkali are still present in the solution when the end point is reached. The acid and alkali are present partly as ions and partly in undissociated form. The magnitude of the errors depends on the p_H of the end point, that is on the indicator used, on the volume of fluid at the end of the titration, and on the dissociation constants of the acid and alkali involved.

If, for instance, we titrate 10 cc. of $N/10$ acid, diluted to 90 cc., with $N/10$ alkali, and as end point use the point where methyl orange just begins to change colour (p_H 3)—we shall have at the end point 99 cc. of a p_H of 3 or 99 cc. $N/1000$ acid, corresponding to approximately 0·1 cc. N acid. If we titrate to p_H 4 the error will be only 0·01 cc. of N acid.

If we titrate the 10 cc. of $N/10$ acid directly with $N/10$ alkali to p_H 3, the error will be 0·02 cc. N acid.

For this error due to the H ions present in the solution at the end point of the titration we may give the following formula:

$$H^{\cdot}\text{-error} = C_{H^+} \times v \text{ cc. } N \text{ solution}$$

where C_{H^+} is the $H^{\cdot}$-concentration and v the volume at the end of the titration.

For the OH-ion-error we have the analogous formula:

$$OH'\text{-error} = C_{OH^-} \times v \text{ cc. } N \text{ solution}$$

where C_{OH^-} is the OH'-concentration at the end of the titration.

These two errors therefore depend on the p_H of the end point and on the volume. To keep both of them as small as possible one would have to titrate to p_H 7.

If we titrate to a p_H on the acid side of p_H 7, then the OH-ion-error is small and the H-ion-error large, and if the end point is alkaline then the OH-ion-error will become large though the H-ion-error is small. If these were the only errors with which to reckon the best procedure would evidently be to titrate to p_H 7 where both errors are small. But this is often impossible because what we titrate is only the dissociated alkali and acid, and if at the end point we have undissociated acid or alkali left an error will arise. How much undissociated acid and alkali we have at a certain p_H depends on the dissociation constants of the substances in question.

Bjerrum gives for this error the following formula:

$$\text{relative acid-error} = 10^{p_{\text{acid}} - p_T} \times \text{the titrated amount of acid,}$$

where $p_{\text{acid}} = -\log K_{\text{acid}}$; K_{acid} being the dissociation constant of the acid and p_T the p_H of the end point.

The formula for the error due to undissociated alkali is:

$$\text{relative alkali-error} = 10^{p_{\text{alk}} - (14 - p_T)} \times \text{the amount of titrated alkali,}$$

where $p_{\text{alk}} = -\log K_{\text{alk}}$; K_{alk} being the dissociation constant of the alkali.

The magnitude of these errors therefore depends on the dissociation-constant of the acid and alkali and of the p_H of the end point.

If one wants the error not to exceed 0·1 %, one must select the p_H of the end point so that it is 3 p_H-units to the alkaline side of p_{acid}, as defined above, or 3 p_H-units to the acid side of p_{alk}.

If for instance one wants to titrate acetic acid and keep this error below 0·1 % then, because the dissociation constant of acetic acid is $10^{-4\cdot 75}$, p_{acid} is 4·75, and we must titrate to an end point of p_H $4\cdot 75 + 3 = p_H$ 7·75. At this point the $H^{\cdot}$-error and the OH'-error are also small, and we are therefore able to titrate acetic acid accurately with an indicator which gives an end point of p_H 7·75 or more alkaline as long as the end point does not differ so much from p_H 7 that the OH'-error is appreciable.

But if we want to titrate H_2S ($K_{H_2S} = 10^{-7}$) the p_{acid} of which is 7·00, we shall, in order to keep the relative acid-error below 0·1 %, titrate to p_H 10. For p_H 10 we get an OH'-error of $10^{-4} \times v$ cc. N solution. If the volume is 100 cc. the error is $10^{-4} \times 100 = 0\cdot 01$ cc. N solution, and to make this error 0·1 % we must have at least 10 cc. N solution in the 100 cc. If the amount of H_2S is less than that, the titration can be performed with an accuracy of 0·1 % only if the volume is reduced.

Generally one may say that if the dissociation constant of either the acid or the alkali involved in a titration forces one to titrate at a p_H higher or lower than 7 in order to keep the relative acid-error low, then the only possible way of keeping down the OH' and the $H^{\cdot}$-errors is to reduce the volume; small amounts of acid or alkali cannot, therefore, be titrated exactly when using diluted solutions of low normality.

If for instance we try to titrate 10 mm.³ N acid with $N/100$ alkali and methyl red as indicator (end point p_H 5) in 100 cc., 10 cc. and 1 cc., then we shall get $H^{\cdot}$-errors which are

$$10^{-5} \times 101 = 0\cdot 001 \text{ cc.} \quad = 1 \text{ mm.}^3 \quad N \text{ acid} = 10\ \%.$$
$$10^{-5} \times 11 = 0\cdot 00011 \text{ cc.} = 0\cdot 11 \text{ mm.}^3\ N \text{ acid} = 1\cdot 1\ \%.$$
$$10^{-5} \times 2 = 0\cdot 00002 \text{ cc.} = 0\cdot 02 \text{ mm.}^3\ N \text{ acid} = 0\cdot 2\ \%.$$

But if we want to keep the end volume small, the amount added during the titration must be small also, and as it is important to keep the end volume nearly constant in spite of variations in the amount of fluid added during the titration, this amount must be small in comparison with the end volume. If for instance we want to titrate in 1 cc., then the amount of titration fluid must be kept below 0·1 cc. This would involve the use of a stronger titration fluid, which in itself is an advantage as in most cases it is more exact and may be kept longer. But we must then be able to add this fluid in amounts smaller than 0·1 cc. with an accuracy of fractions of 1 %. With an ordinary burette this is quite impossible, and even with Bang's microburette the accidental error of reading is easily 0·01 cc. or 10 % of what we want to add.

If we want to titrate in this way we must evidently use a very narrow burette so that 0·1 cc. can be suitably divided and read to say 0·1 mm.³. This is easily done, but it is obviously impossible to add fluid from such a burette in amounts as small as 0·1 mm.³ in the ordinary way by means of a stopcock. Krogh [1908], in his apparatus for micro gas analysis, employed an iron screw working in mercury to move the fluid in the capillary tube of the apparatus. Using this principle it is easy to drive out from a capillary tube as small an amount of fluid as desired, but the fluid will gather at the tip as a drop, and it will therefore be necessary during the titration to dip the tip of the burette into the fluid which is being titrated. As the outcome of such reasoning the apparatus to be described has been constructed.

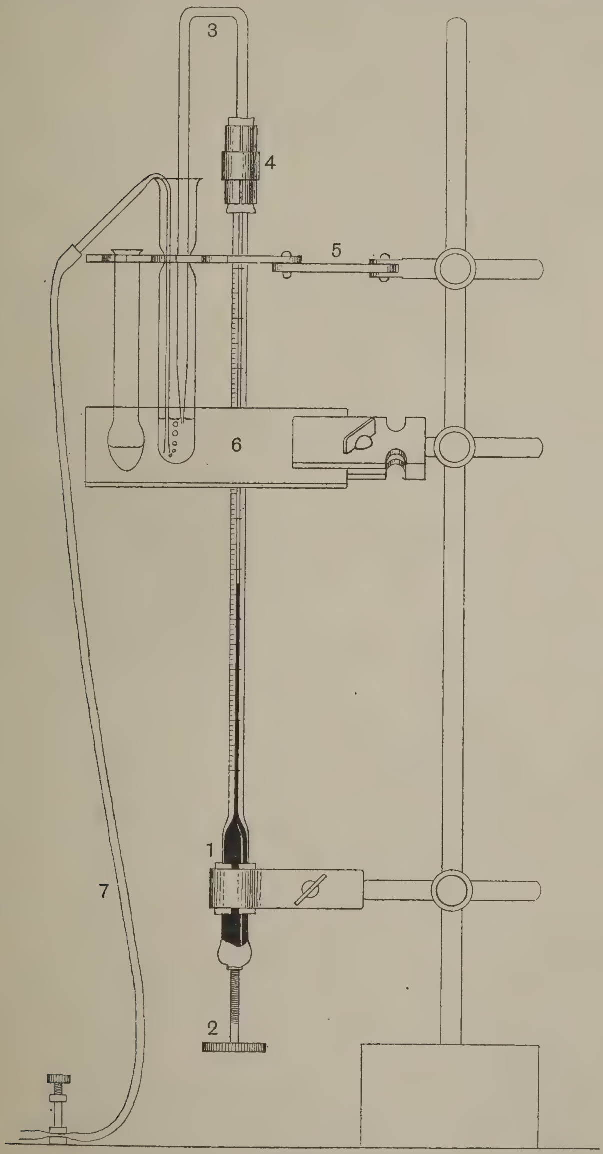

Fig. 1.

The burette[1] consists of a capillary tube about 6–8 mm. thick with a bore of about 0·8–0·9 mm., so that the length of 0·1 cc. is between 15 and 20 cm. It is divided in $\mathrm{mm.}^3$ and calibrated by measuring the length of a weighed drop of mercury at different positions in the tube. At the lower end the tube is widened (1) to take the screw (2), which is of steel and sealed in with picein or sealing wax. This part of the burette is filled with mercury, and by turning the screw the whole of the burette may be filled with mercury. The dimensions of the screw are such that one revolution corresponds to about 4 $\mathrm{mm.}^3$.

As the mercury has to be below the water-solution the burette is mounted as shown in the figure. The tip is then most conveniently made as a loose part (3) and connected with the burette itself by means of a piece of rubber tubing, the connection being strengthened by means of a brass tube cut in two halves and kept together by means of a ring (4). The tip is made of a capillary tube with a bore of 0·8–1·0 mm. and holds more fluid than is used during a titration.

The burette is mounted on a stand which also carries a movable arm (5) arranged to hold the test-tubes used in the titration (two different types are shown in the figure) and a plate of white glass (6) used as background during the titration.

The arm takes three test-tubes, one of which is filled with the fluid used in the titration, one with the indicator used in a buffer solution of the p_{H} wanted as end point, and the third is the tube in which the titration is carried out.

If the solutions used are weaker than $N/10$ the test-tubes used should be made of a resistant glass (Jena or Pyrex).

To fill the burette for the first time with a fresh fluid the tip is removed, the mercury brought up to the top and the burette filled from a pipette by lowering the mercury, and then washed out once or twice. The rubber tube is put on the tip and this is then filled and connected with the burette, care being taken to avoid air bubbles (a single air bubble of 1–5 $\mathrm{mm.}^3$ travelling forward and backward with the movement of the fluid does not interfere with the accuracy of the titration).

When the burette has been used for a titration it can be refilled by dipping the tip in the fluid and lowering the mercury.

The burette is used in the following way: on filling, the meniscus of the mercury is brought a few mm. below the zero point, the tube containing the titration fluid is removed and the tip carefully wiped with a piece of filter paper to remove all fluid adhering to the outside. Just before the titration the mercury meniscus is brought to zero and the small drop now adhering to the tip cautiously removed by touching with filter paper. All readings are made by means of a lens mounted in a holder[2], which is laid against the burette in order to minimise the error due to parallax. In this way the error of the reading may be kept in the neighbourhood of 0·1 mm.^{3}. By means of a pipette a suitable amount (0·5–5 cc.) of the fluid to be titrated is measured out in a test-tube and the indicator added.

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[1] The burette with stand, as in figure, may be obtained from the workshop of the zoophysiological laboratory, Ny Vestergade 11, Copenhagen, at a price of 50 Danish kroner.

[2] As used in the micro-analysis of gases by Krogh.

The amount of the indicator used must, in all titrations which are to be compared, remain the same in order to keep constant the error due to participation of the indicator in the reaction. This error is under these circumstances greater than in an ordinary titration, because the amount of indicator needed to give a sharp end point in such a small volume is comparatively large. For this reason the indicator is best added as 0·1 cc. of a very dilute solution, or a suitable amount of indicator is once for all added to the stock solution.

The tube is now placed in the slot of the arm and raised until the tip of the burette just dips into the fluid. During the titration the fluid is mixed by means of a stream of small air bubbles from a very fine glass tube dipping into the fluid and connected with air pressure (7). The air is filtered through a tube filled with soda-lime to remove carbon dioxide. If air pressure is not available the small amount of air needed may easily be supplied from two 5-litre bottles half filled with weak NaOH-solution and connected to form a gasometer. The bubbling is regulated by means of a clip. By turning the screw of the burette the titration fluid is now added in small amounts till the end point is reached, and the burette is then read again.

To show the accuracy of the method I append a few examples.

By means of a microburette from 5 to 100 mm.^{3} $N/200$ H_2SO_4 were measured into titration tubes. 1 cc. H_2O and 0·1 cc. of a 0·001 % solution of methyl red were added and the tubes titrated with $N/200$ NaOH. The volume in these titrations was at the end between 1·1 and 1·3 cc. If we assume that we are able to hit the p_H wanted (5·5) within 0·5 p_H, we shall at the end of the titration have a p_H varying between p_H 5·25 and p_H 5·75. These two values give the following results for the H$^\cdot$-error:

$$10^{-5\cdot25} \times 1\cdot2 \text{ cc. } N\ H_2SO_4 = 0\cdot0067 \text{ mm.}^3\ N\ H_2SO_4 = 1\cdot35 \text{ mm.}^3\ N/200\ H_2SO_4$$

and

$$10^{-5\cdot75} \times 1\cdot2 \text{ cc. } N\ H_2SO_4 = 0\cdot0021 \text{ mm.}^3\ N\ H_2SO_4 = 0\cdot4 \text{ mm.}^3\ N/200\ H_2SO_4.$$

The accidental error in judging the end point will therefore give rise to variations in the result of ± 0·5 mm.^{3}.

Moreover, we have in each of these titrations four different readings of the burettes on each of which there may be an error of 0·1 mm.^{3}, so that we must expect a maximum error of ± 0·9 mm.^{3}. The mean error will of course be smaller.

The following table shows that two titrations of the same quantity of $N/200$ H_2SO_4 may differ from 0 to 1·3 mm.^{3}.

The table shows, as might be expected, that the results have a systematic error; the quantity of NaOH used is less than the theoretical. This is partly due to the fact that the alkali is not exactly $N/200$ and partly to the H$^\cdot$-error

mm.^{3} *N*/200 acid measured	mm.^{3} *N*/200 NaOH used in titrations		Difference between the two titrations	$\frac{\text{mm.}^3\ \text{NaOH}+1{\cdot}55}{0{\cdot}954}$	
5	2·5	3·5	1·0	4·05	5·05
10	8·0	8·5	0·5	10·00	10·50
20	17·0	17·4	0·4	19·40	19·90
30	26·8	26·9	0·1	29·75	29·85
40	35·9	36·8	0·9	39·30	40·15
50	46·2	46·9	0·7	50·00	50·75
60	56·0	56·1	0·1	60·40	60·50
70	65·5	65·7	0·2	70·35	70·50
80	73·9	75·2	1·3	79·00	80·50
90	84·5	85·1	0·6	90·20	90·80
100	93·1	94·3	1·2	99·20	100·40

and the participation of the indicator in the reaction. If we take these errors into consideration we get the following formula:

$$\text{mm.}^3\ \text{acid} = \frac{\text{mm.}^3\ \text{NaOH} + y}{x}$$

and from the results in the table it is possible to find the values of x and y by the method of least squares.

They are:

$$x = 0{\cdot}954 \text{ and } y = 1{\cdot}55,$$

which means that 100 mm.^{3} acid is equal to 95·4 mm.^{3} NaOH and that the results obtained by the titrations are moreover 1·55 mm.^{3} too low on account of the indicator and $H^{\cdot}$-errors.

In the last two columns of the table are given the values of the titrations corrected in this way. The mean error in a single example of these corrected titrations is 0·52 mm.^{3} *N*/200 solution, which corresponds, if used for instance in an ammonia determination, to 0·000036 mg. N.

In daily use it is of course inconvenient to find the titre of the alkali with respect to the acid by means of least squares. It is best ascertained by an ordinary macrotitration. With stronger solutions the error is negligible, so that the microtitration is sufficient.

If we try to titrate to a point closer to p_H 7 we shall, as has been said, have a smaller $H^{\cdot}$-error. In a series of titrations of *N*/200 acid in a volume of 1 cc. to p_H 6·25 using bromocresol purple as indicator I found a mean error in a single determination of 0·27 mm.^{3} *N*/200 H_2SO_4 or 0·000019 mg. ammonia N.

Using stronger solutions still better results are obtained: working with methyl red and 0·1 *N* solutions, and titrating in 1 cc., I found a mean error of 0·2 mm.^{3} and in 5 cc. of 0·3 mm.^{3}.

This accuracy is sufficient for most purposes, but if still greater accuracy is wanted it will be quite possible to get a little further. The burette may easily be made to measure out still smaller fractions of 1 mm.^{3}—the difficulty will be to observe the change in colour in the small volume required in such cases, but I think that it will be possible to titrate in a volume of about 0·1 cc. which would increase the accuracy to a considerable extent.

As examples of the practical use of the apparatus some methods for the determination of several blood constituents will be published shortly.

SUMMARY.

1. In a brief discussion of the theory of titration the influence of total volume is considered, and it is shown that many titrations can be performed with advantage as microtitrations.

2. An apparatus for microtitration and its use is described. The apparatus allows the addition of fluid from a burette in accurately measured quantities down to 0·1 mm.^{3}.

I wish to express my sincere gratitude to Prof. Krogh for his kind interest and helpful advice during the work.

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Krogh (1908). *Skand. Arch. Physiol.* **20**, 279; or in Abderhalden, Biochem. Arbeitsmethoden, **8**, 1918.

XLII. THE DETERMINATION OF UREA IN 0·1 cc. OF BLOOD BY MICROTITRATION.

By POUL BRANDT REHBERG.

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(*Received February 27th, 1925.*)

Both in the laboratory and in the clinic determinations of blood-urea at short intervals of time are often required, and it is difficult to make them because of the amount of blood needed.

By means of the method of microtitration described in the preceding paper it is possible to overcome this difficulty.

The method to be described is a modification of the Van Slyke method, depending on conversion of the urea into ammonia by means of urease and subsequent aeration of the ammonia into acid and titration of the excess of acid.

The following stock reagents are needed: $N/30$ solutions of H_2SO_4 and NaOH (and if uraemic blood is to be expected $N/10$ solutions); a 0·005 % solution of methyl red[1]; octyl alcohol; saturated K_2CO_3 solution; a 10 % solution of a urease preparation (freshly prepared each day) in water, or, better, in a buffer solution at p_H 7 (Sørensen phosphate buffer p_H 7 diluted 20 times)[2].

The determination is carried out in the following way:

0·1 cc. of blood is measured out in a test-tube in which have previously been placed 0·5 cc. of water and a trace of powdered sodium oxalate. The pipette is rinsed twice with the water and 0·1 cc. of the urease solution is added. The tube is closed, and after the lapse of the usual time (10 mins. at 40°, 30 mins. or more at room temperature) 0·5–1 cc. of saturated K_2CO_3 and a drop of octyl alcohol are added, and the tube is now ready for aeration.

The titration tubes, about 12 × 1·6 cm., which must be flat-bottomed and of resistant glass, are in the meantime prepared as follows: 0·1 cc. $N/30$ H_2SO_4 is measured from a microburette into a titration tube, 4 cc. of water (best redistilled from acidified distilled water), a drop of octyl alcohol, and 0·1 cc. of the indicator solution are added. If a microburette is not used 1 cc. of $N/300$ H_2SO_4 (diluted fresh every day from the $N/30$ solution with a weak indicator solution) is measured out with an accurate pipette and 3 cc. water and octyl alcohol are added. The test-tubes are then connected as shown in the figure, a loose plug of cotton-wool being placed in the tubes between test-tubes and titration tubes, and a current of air, freed from ammonia and carbon dioxide by means of H_2SO_4 and soda-lime, is passed through them.

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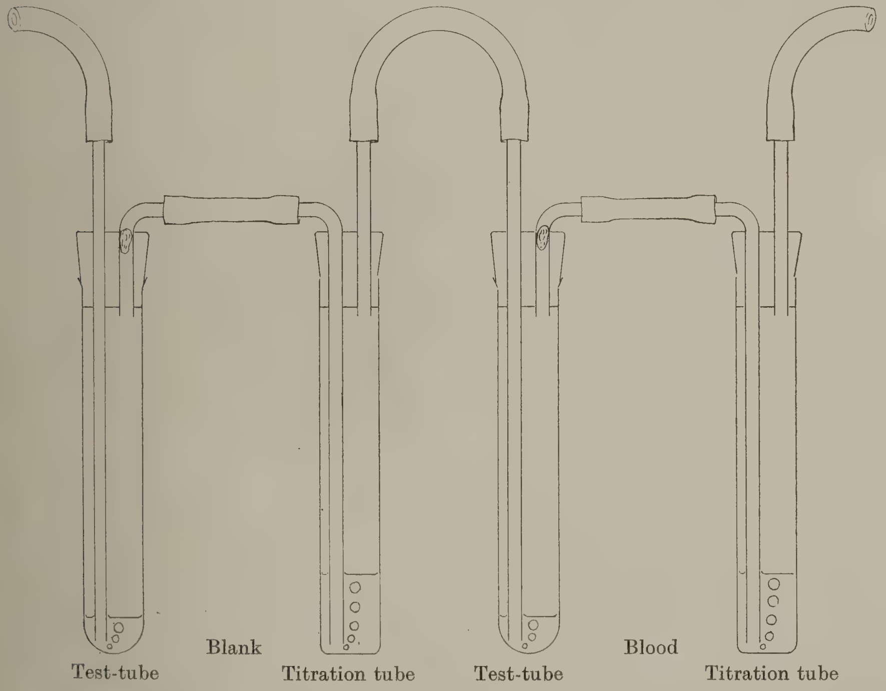
[1] Instead of methyl red a 0·01 % bromocresol purple solution may conveniently be used as indicator—it gives a sharper end point, but it involves the necessity of using solutions absolutely free from carbon dioxide.

[2] 3·9 cc. of a 0·9078 % KH_2PO_4 solution + 6·1 cc. of a 1·1876 % Na_2HPO_4 solution diluted to 200 cc.

The time required for aeration depends of course on the rate of the air current, but I have found it possible to drive over all ammonia in 30–45 mins. One or two blank experiments must be run at the same time.

After disconnection the contents of the titration tubes are titrated by means of the microburette with $N/30$ NaOH, as described in the preceding paper. The difference between a blank and a blood-tube multiplied by the titre factor indicates how many mg. urea are contained in 100 cc. of blood.



Example: 100 mm.^{3} $H_2SO_4 = 97{\cdot}3$ mm.^{3} NaOH

$$\text{Titre factor} = \frac{100}{97{\cdot}3} = 1{\cdot}028.$$

Blank $= 95{\cdot}1$
Blood $= 69{\cdot}7$

$25{\cdot}4 \times 1{\cdot}028 = 26{\cdot}1$ mg. urea per 100 cc. blood.

This method has in my hands given a mean error of ± 0·7 mg. urea on a single determination, but since it is easy to obtain duplicate analyses it allows of a determination with an accuracy of about 0·5 mg.

As examples I shall give the following experiments:

1. Urea solutions of known concentration:

Urea, mg. per 100 cc.	Found
28·95	29·4
	28·5
	28·2
115·8	115·5
144·75	144·9

2. Blood urea:

	Time	mg. per 100 cc.
Fasting	9.30	21·2
		21·2
	10.00	21·0
		21·3
2 eggs eaten	10.15	
	10.50	21·5
		21·0
	11.25	21·5
		21·2
	12.10	25·8
		25·3

3. Blood urea:

	Time	mg. per 100 cc.
	1.45	18·8
		19·7
20 g. urea taken in water	1·52	
	2.10	36·8
		37·3
	2·42	46·2
		47·6
	3·25	56·0
		56·3

XLIII. THE SURFACE TENSION OF GELATIN SOLUTIONS.

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(Received February 10th, 1925.)

In the course of some experiments on a vegetable protein, a marked variation of the surface tension of its solution with changing hydrion concentration was observed. Reference to the literature showed that beyond the well-known surface tension lowering effect of proteins and other colloids, the few observations that have been made have usually taken no account of the hydrion concentration.

It was therefore decided to investigate the effect of the p_H on the surface tension of gelatin solutions, in order to see if the surface tension varied in a similar manner to the other physical properties with change of p_H. Gelatin was used as it can be easily obtained in a relatively pure state and its other physical properties are well known.

Reynolds [1921] gave the value 71·1 dynes/cm. for a fresh 0·5 % gelatin solution as determined by the capillary rise method; this fell to 65·5 dynes/cm. on standing 24 hours, which he attributed to increased surface concentration; he also determined the interfacial tension of gelatin solution against benzene by the drop method.

Mardles [1924] determined the surface tension of gelatin in water and other solvents at various temperatures. The only investigation taking account of the p_H that we can find is a recent paper by Davis, Salisbury and Harvey [1924], who studied various commercial gelatins and gelatin rendered ash-free by Loeb's method, using the drop number method of determining surface tension. Different curves were obtained for various gelatins, the most marked variations with p_H being shown with the ash-free gelatin. This gave a maximum surface tension at about p_H 3·4. They also studied the effects of concentration, time and temperature at constant p_H. No explanation was advanced by them of the changes observed.

The surface tension between the particle and the medium, as an important factor in the stability of colloids, is considered in the theories of Bredig [1901], Donnan [1901], Freundlich [1922], von Weimarn [1911] and others; further references to the literature are given in the *Fifth Report on Colloid Chemistry* [1923].

At the isoelectric point the charge on the particle is a minimum, hence, according to the Lippman effect, the surface tension should be a maximum. Burton and Wiegand [1912] have, however, shown that an electrical charge does not affect the surface tension *per se*, but in general acts so as to oppose the effect of the surface tension.

Loeb [1922] has shown that Donnan's theory of membrane equilibrium explains the colloidal properties of gelatin, and in particular the membrane potentials. Thus we should expect to find some relationship between the P.D.-p_H curves and the surface tension-p_H curves for gelatin.

EXPERIMENTAL.

The measurement of the surface tension was made by Sugden's maximum bubble pressure method [1922, 1924]. This was found to be convenient, rapid and reasonably accurate, besides being unaffected by vibration.

Reynolds [1921] has shown that a dynamic method is desirable for colloids, otherwise the surface tension changes with age owing to increasing concentration in the surface layer; hence the surface tension by a static method falls on standing.

The apparatus was calibrated with pure benzene and was accurate to within 0·3 % for pure liquids, but probably less accurate for colloidal solutions.

When not in use the apparatus was kept filled with either nitric or chromic acid; it was washed with tap water and distilled water before use, and between each determination it was washed with warm nitric acid, and subsequently with tap and distilled water. Similarly all vessels used were pickled in chromic acid, then washed with tap and distilled water and drained dry. These precautions were necessary to ensure freedom from grease.

The gelatin used was Nelson's Gold Label powdered, of an original ash content of 1·5 % and p_H about 5–6. To render it ash-free a modification of Loeb's method of washing was adopted as being more convenient. 15 g. of powdered gelatin were soaked for 24 hours with one change in 500 cc. of a sodium acetate-acetic acid buffer mixture of p_H 4·7 and concentration $N/50$, then for a further 24 hours with distilled water with one change. The temperature was kept below 5° during the soaking. The gelatin was finally washed by decantation and at the pump on a Buchner funnel with 2–3 litres of distilled water, keeping the temperature as low as possible, usually below 8°. The washed gelatin was dissolved in about 300 cc. of distilled water by immersing the beaker in a water-bath kept at 52° for half-an-hour exactly, in which time the gelatin completely dissolved and the temperature of its solution rose to 50°. The warm solution was filtered to remove any undissolved particles, and the concentration was determined by evaporating a portion in a platinum dish in a steam oven for 12 hours by which time the weight had become constant. The dried gelatin in the dish was ashed. The ash content by this method was found to be between 0·08 and 0·13 % on the dry gelatin, which is equivalent to a salt concentration of about $M/30{,}000$, calculating as $Ca_3(PO_4)_2$, in a 1 % solution of gelatin..

The gelatin solution was just remelted in the 52° water-bath and diluted to make the 2 % stock solution, about 500 cc. being obtained. This also set to a gel and each time before use it was just melted in the water-bath, then rapidly cooled to 25° and the required amount withdrawn in a pipette calibrated for that temperature. 15 cc. of the 2 % gelatin solution were added to 15 cc. of solution containing the requisite amount of $N/10$ acid or alkali and distilled water, contained in a conical flask fitted with a rubber bung washed free of grease with spirit. A drop of toluene was added as a preservative, as this was found to have no effect on the surface tension. The solutions were kept for 24 hours in an oven at 25° before determination, in order to allow complete equilibrium to be established. The surface tension was then determined at 25°. In the case of the determinations at 40°, the solutions were only kept an hour at that temperature before determination.

In view of the effect of pretreatment of the gelatin on its physical properties, the above routine was strictly adhered to, although it was later found that the surface tension does not vary with time to an appreciable extent once equilibrium has been established, except in the case of isoelectric gelatin.

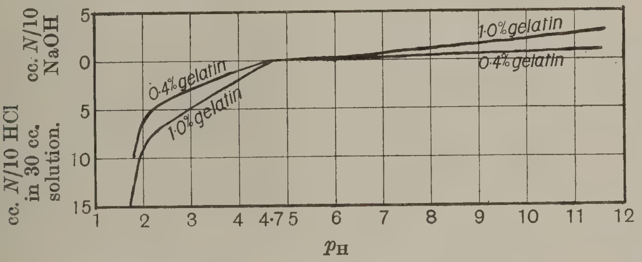

Fig. 1. Titration curve of 0·4 % and 1·0 % gelatin.

THE EFFECT OF HYDROGEN-ION CONCENTRATION.

The hydrogen-ion concentrations were determined colorimetrically, using Walpole and Sörensen's standards and a Cole and Onslow's comparator, and were accurate to 0·1 p_H. The titration curve, cc. $N/10$ acid or alkali in 30 cc. against p_H, was plotted as in Fig. 1. The results above p_H 7·5 were only approximate.

In the determination of the surface tension by Sugden's bubbler, small jets giving a pressure difference of between 7 and 13 cm. alcohol were used, and the bubble rate was usually one bubble per 2–3 seconds on the small jet. The mean of 5 or 6 readings at various bubble rates of about that order was taken as the required value. According to Sugden too slow a rate would give lower values as well as allowing minor leaks in the apparatus to take effect. Too fast a rate (greater than 1 per second) led to excessive frothing and too high values. The determination of the surface tension of the isoelectric gelatin was found the most troublesome. As will be seen from the time curves in Fig. 4, 1 % gelatin p_H 4·7 gradually flocculated in a varying time with different samples, no doubt owing to different ash content or absorbed CO_2 or NH_3

from the air; the surface tension therefore was not determined within 24 hours but after flocculation had occurred, usually in 36–48 hours, though sometimes longer. Owing to the presence of solid particles, a permanent froth was formed which added to the difficulty of determination, and also the values fell on prolonged bubbling, again possibly owing to absorption of CO_2 or NH_3 from the air passing through. From the sharpness of the curves at p_H 4·7 in Fig. 2 this fall of surface tension is easily understood.

The results for 1 % gelatin in presence of HCl, H_2SO_4 or NaOH at 25° and for gelatin in presence of HCl or NaOH at 40° are shown in Fig. 2, all showing a uniform sharp variation of surface tension with p_H, particularly on the acid side.

In view of recent work by Knaggs, Manning and Schryver [1923] on the further purification of gelatin by electrolysis and recrystallisation, a sample of gelatin of ash content 0·08 % was electrolysed according to their method.

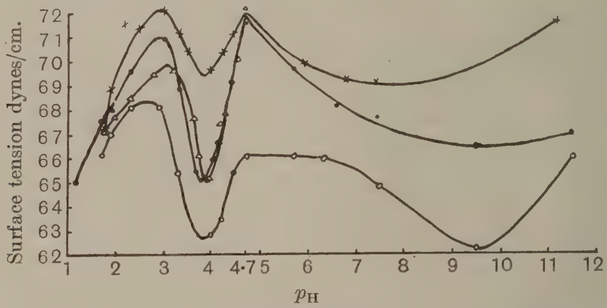

Fig. 2. Variation of surface tension with p_H of 0·4 % and 1·0 % gelatin solutions at 25° and 40°.

× 0·4 % gelatin in presence of HCl or NaOH at 25°
● 1·0 % ,, ,, ,, ,, 25°
○ 1·0 % ,, ,, ,, ,, 40°
△ 1·0 % ,, ,, H_2SO_4 at 25°

We are indebted to Mr R. W. W. Sanderson, of the General Electric Co., for carrying out this electrolysis. The resulting gel was ash-free and an attempt was made to recrystallise it from a 1 % solution, but possibly owing to absorption of CO_2 or NH_3 from the air, it did not flocculate completely and the recrystallised gelatin was difficult to separate either by centrifuging or filtering. The separated gelatin was redissolved, giving a 0·8 % stock solution. The surface tension of the resulting 0·4 % gelatin in presence of HCl or NaOH at 25° is also shown in Fig. 2.

As electrical facilities were not available on the spot, it was not possible to flocculate electrically according to the latest method of Knaggs and Schryver [1924].

THE EFFECT OF NEUTRAL SALTS AND NON-ELECTROLYTES.

As typical salts of uni- and bi-valent acids $NaNO_3$ and Na_2SO_4 were used, and as a typical non-electrolyte glucose (99 % pure). These determinations were carried out at p_H 3·3, and the solutions were prepared either by directly weighing out the required amount of salt or sugar and dissolving in 4 cc. $N/10$ HCl + 11 cc. distilled water, or else by making up a solution and diluting so that 11 cc. of it together with 4 cc. HCl gave the final required concentration in 30 cc. The surface tension at p_{H} 3·3 with no additions was the mean of three separate determinations. The p_{H} of the solutions was found to fall gradually from p_{H} 3·3 to p_{H} 3·15 with increasing salt concentration up to $M/16$.

As will be seen from Fig. 3 the surface tension falls with increasing salt concentration and more for Na_2SO_4 than for $NaNO_3$. The effect of the fall of p_{H} would tend to raise the surface tension, as can be seen by consideration of the curve for gelatin in HCl in Fig. 2. The non-electrolyte glucose had very little effect even in concentration $M/4$—the slight lowering being probably due to the ash content of the glucose.

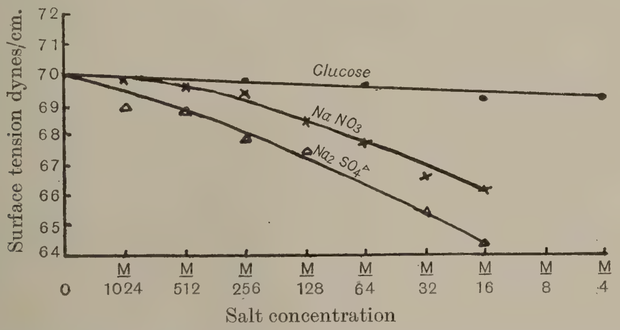

Fig. 3. Effect of neutral salts and of glucose on surface tension of 1 % gelatin in presence of HCl at p_{H} 3·3 and 25°.

The Effect of Time.

Although the results in Figs. 2 and 3 were all obtained under uniform conditions, the effect of time on the surface tension was studied in one or two cases. The determinations were started immediately the gelatin had been dissolved and filtered.

Equilibrium was apparently reached at p_{H} 3·3 in an hour or so, and then remained constant within experimental limits for the several days over which it was observed. With isoelectric gelatin, the surface tension rose gradually from a low value of about 66 dynes/cm. up to 71·5 in 72 hours, *i.e.* approaching that of water, as flocculation proceeded. In other cases where no previous readings of the surface tension were taken it had risen very nearly to 72 in 36–48 hours, when complete flocculation had occurred. The results are shown in Fig. 4.

Discussion of Results.

Considering first the surface tension-p_{H} curves at 25° in Fig. 2, it will be seen that the effect of decreasing the concentration of gelatin from 1 % to 0·4 % is to increase the surface tension as would be expected. All the curves at 25° show a maximum at p_{H} 4·7, as was expected, values approaching that of water being obtained. This corresponds to a very low P.D. and solubility and a maximum of neutral un-ionised particles at the isoelectric point, but presumably there is some dissociation there, causing a very slight lowering of surface tension. The curve at 40° differs from those at 25° in that the surface tension at the isoelectric point is considerably lower than that of water at 40° and that there is no sharp maximum. There is evidently considerably more gelatin in solution at that temperature, and presumably more dissociation into electrically equal amounts of positive and negative ions. Pauli [1921] has shown from mathematical considerations that this would be expected.

The 40° curve shows a marked flattening between p_{H} 4·6 and 6·0, probably due to the above increased dissociation; the dissociation constants of positive and negative dissociation may increase at different rates with temperature rise, and from the expression $C_{\mathrm{H}} = \sqrt{\frac{K_a K_w}{K_b}}$ for the hydrion concentration of the isoelectric point, where K_a, K_b and K_w are the dissociation constants of positive and negative dissociation of the protein and of water respectively, we should expect a small change in the p_{H} of the isoelectric point with temperature. Our results are not accurate enough to determine this, beyond the marked flattening, except that, whereas at 25° the surface tension of isoelectric gelatin was found to be practically independent of concentration, as is to be expected theoretically from the calculations of Pauli, Michaelis and Sörensen, at 40° the surface tension at p_{H} 4·7 was found to vary with the concentration values:

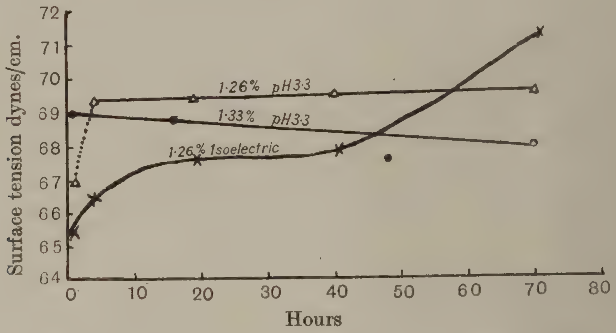

Fig. 4. Effect of time on surface tension of gelatin at 25°.

1 % gelatin, surface tension = 66·1 dynes/cm.
2 ,, ,, ,, ,, = 64·4 ,,
6 ,, ,, ,, ,, = 62·3 ,,

This may be an indication that the isoelectric point at 40° is not exactly at p_{H} 4·7.

All the curves for gelatin in presence of HCl show a sharp point of minimum surface tension at p_{H} 3·8, and for gelatin in presence of H_2SO_4 at p_{H} 3·9–4·0. This agrees well with Loeb's calculation and determination of the maximum P.D. of 1 % gelatin at p_{H} 3·8–4·0. The P.D. of gelatin in presence of H_2SO_4 is only two-thirds that of gelatin in presence of HCl, though no difference in the lowering of surface tension was found by us, and, as will be seen later from the effect of neutral salts, the surface tension lowering cannot be considered as entirely dependent on the P.D. Loeb further shows that the P.D. decreases steadily from this maximum value at p_H 3·8–4·0 down to p_H 1. We, however, find a well-marked maximum value again for the surface tension at about p_H 3·0, after which the surface tension again falls rapidly with increased acid concentration. Davis, Salisbury and Harvey show a similar maximum about p_H 3·4. This may be due to a change in the gelatin molecule with formation of "acid protein." This conclusion is supported by the titration curves shown in Fig. 1, which agree with those of other workers in showing a sharp change of direction about p_H 2·5, indicating increased combination of acid. Henderson, in Osborne's *The Vegetable Proteins* (1923), shows that at p_H 1–2, and again at p_H above 10, the titration curves of proteins no longer agree with those required by theory and attributes this to changes in the molecule in more concentrated acid or alkali. Pauli finds that other proteins with strong acids are most sensitive to coagulating agents such as alcohol at a p_H of about 3·0. According to Bredig, Burton [1923] and others, a high surface tension would be conducive to precipitation. Surface tension may show this change in the molecule before the titration curves do, as it is obviously very sensitive to small changes; hence our maximum occurs earlier, about 2·8–3·0. On the other hand, the drop in surface tension after this maximum may be due to increased dispersion brought about by peptisation. Further experiments are necessary to find out what is occurring in this region.

On the alkaline side a minimum surface tension is observed at a p_H of about 9–10, which agrees with Loeb's observed and calculated values for maximum P.D. and also for swelling of gelatin in NaOH solutions.

The results obtained by the addition of neutral salts to gelatin in presence of HCl are entirely unexpected. The Donnan theory of membrane equilibrium demands that such addition should depress the P.D. of the gelatin and the more so the greater the concentration of added salt. Moreover, the depression of the P.D. caused by a salt of a univalent acid, such as sodium nitrate, should be only two-thirds that produced by equimolar amounts of a salt of a bivalent acid, such as sodium sulphate. If surface tension is influenced by the P.D., depression of the latter should lead to a rise in surface tension. We find, however, as will be seen from Fig. 3, that the addition of a neutral salt produces a depression of the surface tension very nearly proportional to the concentration of the added salt. Curiously enough the depression produced by a salt of a univalent acid is approximately two-thirds of that produced by a salt of a bivalent acid when the concentration of the added salt exceeds $M/256$. Berczeller [1917] found that the addition of salts lowered the surface tension of protein solutions. Davis, Salisbury and Harvey similarly found that the surface tension of ash-free gelatin was much higher than that of a commercial gelatin containing mineral impurities.

The addition of a non-electrolyte, such as glucose, does not alter the surface tension of the sol except for a slight depression due to the mineral impurities of the glucose. Since it can play no part in the Donnan equilibrium, this is to be expected.

Recent work by Manning [1924] on electrically flocculated isoelectric gelatin has shown that this may be peptised or dispersed to a stable gel by very small concentrations of acid and alkali, whilst relatively larger concentrations of neutral salts will produce the same effect. Evidently the addition of neutral salts tends to increase dispersion of the gelatin, making relatively more gelatin in the sol form. This affects the surface tension, causing a lowering.

The surface tension-time curves in Fig. 4 show that the rapidly cooled gelatin solution has a low surface tension, which at p_H 3·3 rises within an hour or so to its equilibrium value, which it then maintains within experimental limits over the period observed. Gelatin at the isoelectric point similarly starts at a low value, which rises gradually as the solution changes over to the gel form and flocculates, when a maximum surface tension near that of water is reached. Smith [1919] has shown by following the mutarotation that there is an equilibrium between the sol and gel forms of gelatin between the temperatures of 15° and 35° depending on the temperature.

Evidently then anything which increases the amount of the sol form in the solution, whether it be rise of temperature, presence of acid or alkali, or neutral salts, will cause an increased lowering of surface tension. Donnan's membrane equilibrium theory apparently accounts quantitatively for the ionisation of ash-free gelatin and for the membrane potentials observed by Loeb, but as far as surface tension is concerned, the other factors, such as peptisation of the neutral gelatin particles by neutral salts, and strong acids in higher concentration, and the physical state of the gelatin as influenced by temperature and time must also be taken into account. Further, the effect of strong acids at concentrations giving a p_H less than 3 is more marked on the surface tension than on any other property, though as to what actually occurs further investigation is required. It will be seen then that it is not possible in our present state of knowledge of the relationship of the surface tension of solutions to the many factors affecting it, to make any generalisations, except that lowering of surface tension is coincident with increased dispersion and stability of the colloid, and that a high surface tension tends to coagulation. As a means of determining the isoelectric points of proteins whose other properties are difficult of determination, the method may be useful, provided distinction is possible between the true isoelectric point and the maximum produced at a higher acid concentration.

Further work with other proteins is being undertaken in this direction.

SUMMARY.

1. No direct quantitative relationship could be traced between the surface tension of a colloidal solution and the potential difference between the particles and the medium.

2. The surface tension of gelatin solutions is found to be a maximum at the isoelectric point (p_H 4·7); a minimum at p_H 3·8–4·0 on the acid side, and in the neighbourhood of p_H 9 on the alkaline side. A second maximum is found at p_H 2·8–3·0, after which the surface tension decreases steadily. It is suggested that this second maximum marks the point at which the formation of "acid-gelatin" begins.

3. Increase of temperature and addition of electrolytes both cause a lowering of the surface tension of gelatin solutions. This indicates that increased solubility or peptisation of the gelatin lowers the surface tension.

4. The surface tension of gelatin solutions is little affected by time after equilibrium is attained, which occurs in a few hours at 25°, except in the case of isoelectric gelatin, whose surface tension slowly rises nearly to that of water as flocculation proceeds.

We are indebted to Dr A. L. Stern and Dr A. Slator for advice during the course of this investigation, and to the Directors of Bass, Ratcliff and Gretton, Ltd., for permission to publish our results.

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XLIV. THE OCCURRENCE OF DIASTASE IN THE CEREBRO-SPINAL FLUID.

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(*Received February 25th, 1925.*)

The earliest investigations into the chemistry of cerebro-spinal fluid were carried out on fluid obtained from cases of hydrocephalus and spina bifida, and so we find that as early as 1891 Toison and Lenoble [1891] reported that they found the cerebro-spinal fluid of a hydrocephalic to have a slight amylolytic power. Several years later Cavazzani [1896] observed similar results in two such cases. Galletta [1908], who examined eleven normal cases, only found amylase in two of them. More recently Mestrezat [1912] stated that in two normal fluids he found just a trace of diastase, and in his book he comes to the conclusion that normal cerebro-spinal fluid has no amylolytic power or only very slight traces of it. Stocks, however, has stated [1916] that he had succeeded in demonstrating amylase in every fluid of the body which he had examined, including the cerebro-spinal fluid.

It is important to note that the term "normal" as applied to cerebro-spinal fluid must be considered merely relative, for the mere fact of there existing a reason for withdrawing some of the fluid from the living person makes the case a pathological one. Again, post-mortem fluid is known to differ from that in the living, so that observation on post-mortem cerebro-spinal fluid cannot be considered normal. It is only by considering a series of fluids taken during life and post-mortem that it is possible to arrive at some conclusion as to what the normal is likely to be. Both Galletta and Mestrezat (*vide supra*) define their "liquides normaux" as obtained from "sujets chirurgicaux."

The cases in the investigation about to be described fall into two groups. Firstly those in which the cerebro-spinal fluid was taken during life and secondly those in which the fluid was taken at known intervals after death. In many of the cases in the latter group a specimen of blood was taken at the same time for purposes of comparison.

Method. The method used for estimating the diastatic content of the cerebro-spinal fluid was a slight modification of the method for other body fluids, described in conjunction with Dodds [1924].

The following solutions are required:

1. Buffer solution. This is made by mixing 15 cc. of Sörensen's phosphate solution A with 85 cc. of solution B. The resulting solution should have a p_H of 6·1. Solution A: 11·876 g. of $Na_2HPO_42H_2O$ dissolved in 1 litre of boiled

distilled water. Solution B: 9·078 g. of KH_2PO_4 dissolved in 1 litre of boiled distilled water. The solutions are kept in paraffin-coated bottles, and a drop of toluene is added to prevent the growth of moulds.

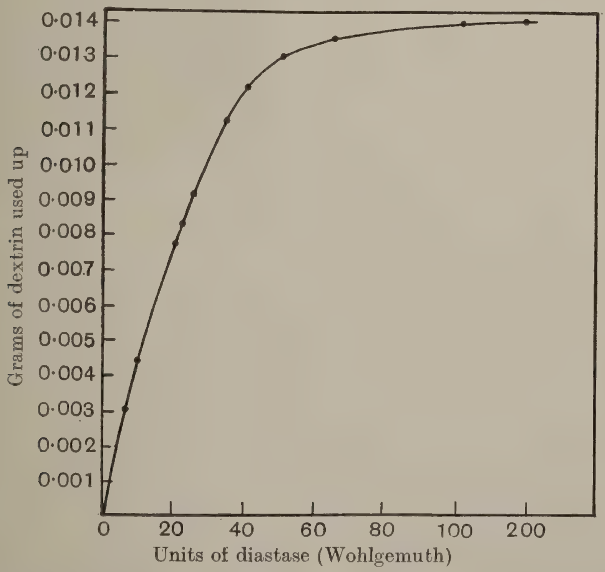
2. Standard dextrin solution: 0·75 g. of pure erythro-dextrin is dissolved in a small volume of boiled distilled water, and the volume is made up to 100 cc.

3. Iodine solution:

N/10 iodine in potassium iodide solution ...	20 cc.
Saturated ammonium sulphate solution ...	500 ,,
Distilled water	1000 ,,

Procedure. 1·5 cc. of cerebro-spinal fluid are pipetted into a tube and 6 cc. of buffer solution are added. The mixture is well shaken. 0·5 cc. of this buffered mixture is pipetted into a test-tube, graduated at 30 cc., and 1·0 cc. of the standard dextrin solution added. After thorough shaking the tube is incubated in a water-bath at 37° for half-an-hour. Whilst the incubation is proceeding the standard is prepared. This is made up by pipetting 1·0 cc. of standard dextrin into a tube graduated at 30 cc.

When the incubation period is complete the tube is removed from the water-bath, and 15 cc. of the iodine solution are added to it and to the standard. The volume of each is then made up to the mark, mixed and compared in the colorimeter, the standard being set at 20 mm.



Small-scale curve to show relation between units of diastase and grams of dextrin used up.

The amount of dextrin present in the unknown is calculated by the familiar colorimetric ratio—that is: $\frac{20}{X} \times Y$; where X is depth in millimetres of unknown, and Y is the quantity of dextrin in the standard. Since 1 cc. of 0·75 % dextrin solution—that is, 0·0075 g. of dextrin—was present before incubation, the amount of dextrin used up can be calculated by subtracting from this the figure obtained in the above calculation. By reference to the curve this can be converted into Wohlgemuth units.

Group I. The cerebro-spinal fluid in this group was taken during life. Twenty-eight cases were examined with the following conditions:

Tabes	6
Syphilis (primary and secondary)	5
Uraemia	3
General paralysis of the insane	2
Tuberculous meningitis	2
Cerebral tumour	2
Cerebral syphilis	2
Encephalitis lethargica	1
Neuritis	1
Meningo-myelitis	1
Acute anterior polio-myelitis	1
Cerebro-spinal meningitis	1
Infective endocarditis	1

In addition, three samples of cerebro-spinal fluid were examined which were obtained by collecting the fluid drawn off before the injection of a spinal anaesthetic.

In the uraemic cases the urea content of the fluids was estimated for diagnostic purposes. These figures will be referred to later.

Results. In all the thirty-one cases except two not a trace of diastatic enzyme was detected. In one case of tabes and one of secondary syphilis only a trace (less than one unit) of diastase was found.

Group II. In the thirty-six cases comprising this group the spinal fluid was collected at known intervals post-mortem. Blood was taken at the same time. Urea estimations were carried out on the specimens to show the presence, if any, of nitrogen retention, for it is reasonable to expect to find in cases showing a high urea content accumulation of other substances, including perhaps the enzyme diastase.

Results. Only seven of these cases were observed to contain diastase in the cerebro-spinal fluid. From the table given below it will be seen that four of the seven show marked urea retention:

		Blood		Cerebro-spinal fluid	
Case	Hours after death	Urea mg. per 100 cc.	Diastase units	Urea mg. per 100 cc.	Diastase units
1	16	284	8	261	8
2	7	90	7	82	2
3	24	44	5	37	3
4	8	104	4	91	2
5	11	101	30	91	3
6	21	28	3	26	4
7	10	—	4	—	1

That the cause of the urea retention is not associated with the appearance of the diastase in the cerebro-spinal fluid will be seen when it is stated that the urea figures for the uraemia cases in Group I were 101, 102 and 143 mg. per 100 cc. No trace of diastase was found in these latter cases where the fluid was withdrawn during life.

DISCUSSION.

It appears that during life, whatever the condition is, it is rare to find diastase in the cerebro-spinal fluid. It will be admitted that the nearest approach to normal fluid was obtained from cases punctured for spinal anaesthesia, and it is very significant that none of these contained any diastase. Moreover, twenty-six of the cerebro-spinal fluids derived from ante-mortem sources yielded similar results, and only two were found to contain any diastase at all, this being under one unit. It is very unlikely, therefore, that diastase is a normal constituent of cerebro-spinal fluid. In all the cases taken within 24 hours after death about 20 % were found to contain appreciable quantities of diastase. The presence of the enzyme was not observed in any case until about 7 hours after death, quite independently of the occurrence of nitrogen retention. It is therefore a post-mortem effect.

SUMMARY.

1. The presence of diastase in the cerebro-spinal fluid of human beings during life is extremely rare.

2. Approximately 7 hours after death roughly 20 % of cerebro-spinal fluids were found to contain definite amounts of the enzyme.

3. The appearance of diastase in the cerebro-spinal fluid is a post-mortem effect occurring after about 7 hours.

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XLV. THE ESTIMATION OF AMMONIA AND UREA IN BLOOD AND URINE.

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(*Received January 16th, 1925.*)

The principles underlying the chemical changes involved in the method here described are exactly those of the well-known Folin macro aeration method. But the objections to the latter are that the apparatus is very cumbersome, and unless extremely well set up is likely to give too low results.

The apparatus I here propose is that designed by Cannan and Sulzer [1924] for the estimation of alcohol in blood. The great advantage of the apparatus is that it is very compact and easy to handle. The estimation takes less time than in the ordinary aeration apparatus; the loss of ammonia and errors due to rubber connections are cut down to a minimum. This form of apparatus can be quite easily used by students in class. The great advantage in time and ease with which the whole operation is carried out makes it desirable that the details of the procedure should be described.

Method for Ammonia in Urine.

In the outer vessel (Fig. 1) place 20 cc. urine. To the inner vessel add 20 cc. $N/10$ H_2SO_4, a few drops of methyl red and enough distilled water to cover the end of the spiral. Insert the inner part in position, then make connections at X with a sulphuric acid washer and at Y with a pump. Raise the cork and deliver into the bottom of the jacket 5 g. anhydrous Na_2CO_3 from a narrow test-tube. Press the cork in tightly, place the apparatus in a bath at 40° and aerate with a very brisk current of air for 20 to 30 minutes. After this time disconnect from the pump and washer, remove the inner vessel and wash this well on the outside. Then, holding the apparatus over a funnel placed in a flask, remove the inner part, transfer the contents to the flask and wash all the parts very thoroughly. Then titrate the contents of this flask with $N/20$ NaOH. The accuracy of the method has been tested by using a standard ammonium sulphate solution (1 cc. = 1 mg. N) and aerating for 30 minutes; the following results were obtained.

mg. N taken		mg. N estimated
10		9·87
10		9·87
10		9·94
10		10·00
	Mean	9·92

The results for the ammonia in urine have been checked by the recently described colorimetric method of Orr [1924].

Aeration method. mg. ammonia N per 100 cc.	Colorimetric. mg. ammonia N per 100 cc.
55·0	56·3
24·2	24·6
16·18	16·18
39·9	41·0
17·5	17·5

Method for Urea in Urine.

The quantities used are 5 cc. of urine (of known ammonia content), 15 cc. of active urease solution prepared by the method of Folin and Wu, and 5 cc. of sodium pyrophosphate solution. The inner vessel contains 10 cc. $N/2$ H_2SO_4, distilled water and methyl red, as in the case of the ammonia determination. The digestion by the urease is allowed to proceed in a bath at 40° for 20 minutes. Then the sodium carbonate is added and the air current passed for half-an-hour. The rest of the estimation is carried out as described before, $N/10$ alkali being used in the titration of excess sulphuric acid.

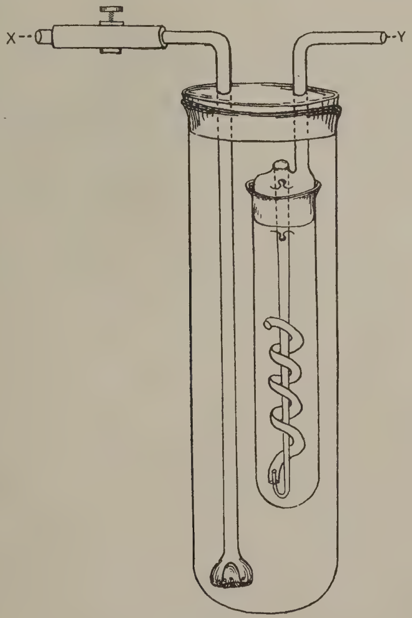

Fig. 1.

These quantities and times have been tested on a standard urea solution of about the same concentration as urine (1 cc. = 10 mg. N).

mg. urea N taken	mg. urea N estimated
100	98·2
100	97·5
100	98·2
100	98·4

Method for Ammonia and Urea in Blood.

A fairly accurate estimation of the urea in blood can be made in a similar way. This is carried out in a smaller form of the apparatus, in which the jacket is 3 cm. in diameter and the inner vessel correspondingly smaller. The volume of blood taken should not be less than 0·6 cc. The proteins are first precipitated by the method of Folin and Wu [1919], and 5 cc. of the clear liquid, which is equivalent to 0·5 cc. blood, are used for the estimation.

I have used 2 cc. $N/100$ H_2SO_4 in the inner vessel to catch the ammonia and titrated the excess acid with $N/200$ NaOH from a burette graduated to 0·02 cc.

For two estimations on a sample of cat's blood I obtained the following values.

Equivalent of blood taken cc.	$N/100$ H_2SO_4 neutralised cc.	mg. urea N per 100 cc. blood
0·5	0·68	19
0·5	0·62	16

The error in this method amounts to not more than ± 4 mg. urea nitrogen per 100 cc. blood.

Although a method for the estimation of urea in 0·2 cc. of blood has been described by Thomas [1924], using $N/400$ acid and alkali, I have been unable to get consistently good results when employing it. This is not difficult to understand, for, if the proteins are precipitated by MacLean's method [1919], the filtrate obtained could not represent more than $\frac{5}{6}$ of the original blood taken, which for a normal blood, containing between 15 and 20 mg. urea nitrogen per 100 cc. blood, corresponds to 0·03 mg. of urea nitrogen. Working with so small a quantity of urea nitrogen, the doubtful quantitative precision of $N/400$ acid and alkali, contained probably in soda glass vessels, to my mind tends to make the method somewhat unreliable.

The extension of Orr's method for the Estimation of Ammonia to the Estimation of Urea in Blood and Urine.

Orr [1924] has recently described a colorimetric method for the estimation of ammonia in urine. I have found the results obtained in this way to agree very closely with those obtained by the aeration method. The estimation is very rapid and, although there are inherent objections to colorimetric methods, it seemed worth while to extend the method to the estimation of urea, since it is usual to have to estimate ammonia and urea in the same sample of urine.

The application of the principles of the colorimetric method to urea estimation was tested, using standard solutions of urea. The details were as follows:

10 cc. of standard urea solution (containing 10 mg. nitrogen per cc.) were pipetted into a 100 cc. graduated flask; 20 cc. of the active urease solution and 10 cc. of pyrophosphate solution were added and the whole made up to

100 cc. The contents of the flask were rapidly transferred to a small conical flask which was very tightly corked and then placed in a bath at 40° for half-an-hour. The flask was then cooled under the tap, the cork raised, and 10 cc. of the solution rapidly pipetted into a 100 cc. flask containing approximately 90 cc. of distilled water; the volume was adjusted to the mark.

The estimation was then carried out with 5 cc. of this solution exactly as described by Orr and using the standard ammonium sulphate (1 cc. = 1 mg. N) for comparison.

This method seems to estimate only 94 % of the total urea and as this figure was very constant with the standard urea solutions, and the values for urea in urine by this method were lower than in the aeration method, it is proposed to make a correction for this in stating results. This low value is no doubt due to inactivation of the urease.

The hydrolysed urea solutions are very easily compared; there is no turbidity due to the enzyme solution and no alteration in the tint of the solution.

If soya-bean meal alone is used the comparison is not so easily made and several times when using the meal I have obtained results much higher than the theoretical value.

Estimation of Urea in Urine Colorimetrically.

10 cc. urine, 20 cc. urease solution, 10 cc. pyrophosphate solution are run into a 100 cc. flask and made up to the mark. This is transferred to a conical flask, corked well and digested in a bath at 40° for half-an-hour. After cooling, 10 cc. of this solution are taken and diluted to 100 cc., as described before. 5 cc. are used for the estimation in the usual way.

Example.

Ammonia N aeration. mg. per 100 cc.	Ammonia N colorimetric. mg. per 100 cc.	Urea N aeration. mg. per 100 cc.	Urea N colorimetric. mg. per 100 cc.	Corrected value for colorimetric. mg. per 100 cc.
55·0	56·3	990	941·5	1000
39·6	41·0	1031	960·0	1021

Estimation of Urea in Blood.

Though the details have been worked out for 0·2 cc. of blood, larger quantities can be used, probably with better results, but for clinical purposes it seems desirable to have a method suitable to this small quantity. If a systematic blood analysis is being made by the Folin-Wu procedure then the estimation is carried out on 2 cc. of the protein-free filtrate.

Method for 0·2 cc. of blood. Place in a small centrifuge tube of about 7 cc. capacity 4·6 cc. of distilled water, run the blood into this from a MacLean 0·2 cc. pipette, washing the pipette out in the usual way. When the blood is laked add 0·6 cc. of 10 % sodium tungstate and then 0·6 cc. of $\frac{2}{3}$ N H_2SO_4 (giving a total volume of 6 cc.). Mix and shake till the typical chocolate colour is obtained and heat gently. Centrifuge the tube till the precipitate is well settled. Then transfer by means of a pipette 5 cc. of the supernatant liquid, which represents $\frac{5}{6}$ of 0·2 cc. of blood, into the outer jacket of the small aeration apparatus described above, add 2 cc. urease extract and 0·5 cc. of pyrophosphate solution. In the inner vessel place 2 cc. of distilled water, two drops of $N/10$ H_2SO_4 and 2 g. of pure phenol. Place the apparatus in a bath at 40°, connect to a pump in the usual way and leave to digest for 10 minutes. Then disconnect, run in 5 cc. saturated Na_2CO_3 solution, replace the apparatus in the bath and aerate for 20 minutes.

Take two 25 cc. graduated tubes; to one which is to be used as the standard add 2 g. of phenol, 4 cc. of distilled water and then 2 cc. of standard ammonium sulphate solution containing 20 mg. nitrogen per 100 cc. Into the other tube empty the contents of the inner tube of the aeration apparatus, wash this tube and spiral with 2 cc. of water and add the washings to the graduated tube. Then to each of the tubes add 5 cc. of sodium hypochlorite, gradually and with shaking, allow to stand for 5 minutes, make each up to the 25 cc. mark and compare at once in a colorimeter with the standard at 30 mm. or 20 mm. as preferred. A stronger standard will sometimes be needed or alternatively the unknown solution can be diluted.

Results. Standard urea solution containing 20 mg. urea N per 100 cc.

Reading for standard ammonium sulphate solution: 20 mg. N per 100 cc. mm.	Reading for the urea solution mm.		Strength of urea solution calculated: mg. per 100 cc.
30	28·7		20·9
30	30·6		19·6
30	31·0		19·0
		Mean	19·8

Results for Blood. A. Using 2 cc. Folin-Wu filtrate ≡ 0·2 cc. blood.

	Standard reading mm.	Blood reading mm.	mg. urea N per 100 cc.
A_1	20	26·6	15
A_2	30	39·6	15
B_1	30	29·6	20

B. Using 0·2 cc. of same blood as used in A_1 and A_2 and precipitating as described.

Standard reading mm.	Blood reading mm.	mg. urea N per 100 cc.
20	29·6	16
30	45·2	15

The colour of these solutions is a clear blue, slightly greener than that obtained with the urine standard, and it is, I think, much more suitable for colorimetric comparison than the yellow of Nesslerised solutions of the same ammonia content. It does not seem possible to use the protein-free filtrate directly in this estimation, for if this solution is digested and then run on to the phenol a green coloration develops on the subsequent addition of the hypochlorite solution.

SUMMARY.

1. Ammonia and urea in urine and blood are estimated by aeration in a simple form of apparatus.

2. Urea in urine is estimated colorimetrically.

3. Urea is estimated in 0·2 cc. blood by combination of the aeration and colorimetric methods.

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XLVI. DETERMINATION OF CARBON MONOXIDE IN BLOOD.

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Carbon monoxide in blood has been determined in several ways. Except in methods like Haldane's [1895], which is colorimetric, and Hartridge's [1912], which is spectroscopic, a combination of two chemical treatments is necessary; namely, the extraction of the CO from the blood and the subsequent determination of the quantity of the gas.

The determination of CO by means of iodine pentoxide has found a wide application, quantities as small as 0·05 cc. being estimable. The method was first applied to blood by Nicloux [1898], who extracted the CO with the vacuum-pump of Gréhant [1894]. Later on nobody seems to have used iodine pentoxide for this purpose. Plesch [1914] and Gad-Andresen [1916] liberated the CO together with the oxygen in the apparatus of Barcroft or Krogh by means of potassium ferricyanide and determined the CO by combustion.

Van Slyke and Salvesen [1919] liberated the CO together with the oxygen and determined the former gasometrically after absorption of the oxygen.

When, however, the CO content of the blood is less than 10 % of its total capacity these methods are lacking the accuracy necessary in physiological and forensic medical work. The method given by Nicloux may give better results, but he does not give any control experiments on the subject.

By combining Haldane's ferricyanide method with the use of I_2O_5 it is possible to reach a greater accuracy, if one can afford to use at least 10 cc. of blood.

1. *Determination of small quantities of* CO.

CO made in the ordinary way from formic and sulphuric acids was analysed by means of the capillary pipette described by Jordan and Schwarz [1920]. As absorbing fluids were used a solution of pyrogallic acid in strong potassium hydroxide for oxygen and carbon dioxide, and a solution of cuprous chloride in strong ammonia and ammonium chloride for carbon monoxide.

A small quantity of this analysed gas was measured in the Jordan-pipette and sucked into an evacuated flask of one litre capacity. Then outdoor air was introduced and mixed with the CO by shaking, a small quantity of water securing a thorough mixing. The resulting gas mixture was then led over I_2O_5 heated to 150°. This I_2O_5 (5 g.) was contained in a **U**-tube (*D*) of Jena glass, being kept in position by small plugs of glass-wool or asbestos. The gas before reaching the I_2O_5 is led first through strong KOH (*A*) and then through strong sulphuric acid (*B*) to be freed from traces of SO_2 and water, which would interfere with the determination of the CO. Tube *C* is a glass spiral to retain traces of sulphuric acid. Ordinary Peligot tubes were used as wash bottles. The iodine liberated is retained in two Peligot tubes (*E* and *F*) containing 0·5 % KI solution, and afterwards titrated with 0·01 *N* thiosulphate from a micro-burette. 1 cc. of 0·1 *N* thiosulphate corresponds to 5·6 cc. of CO.

Before the apparatus is used for determinations the I_2O_5 should be subjected to a current of air devoid of reducing gases at a temperature of about 200° for several hours, until it gives off only traces of iodine at 150°; these traces should be constant in quantity in order that a reliable blank value may be obtained.

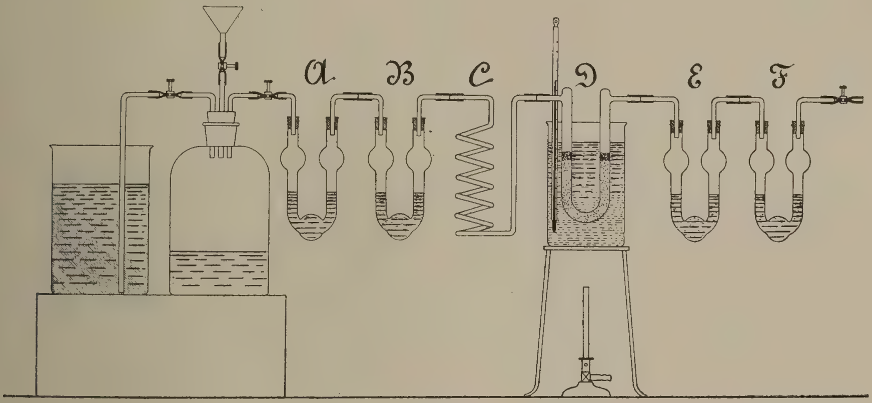

Fig. 1.

Table I indicates amounts of CO taken, as calculated from the values found with the use of the Jordan pipette and those found by titration of the iodine liberated.

Table I.

cc. CO taken	cc. CO found
0·027	0·043
0·060	0·068
0·113	0·122
0·144	0·120
0·149	0·114
0·308	0·319
0·634	0·680
1·21	1·04
1·61	1·31

In the last three determinations the gas was measured by means of a Van Slyke pipette.

2. *The extraction of the* CO *from the blood.*

Van Slyke and Salvesen have pointed out that for extraction of oxygen and CO from blood it is convenient to use ferricyanide together with evacuation. This principle has been applied in the following way. For the extraction a wide-mouthed bottle of 1 litre capacity is used. This bottle is closed by a rubber stopper, through which pass three tubes; two of these are bent at right angles; all three carry rubber tubes, which are closed by screw clamps (see Fig. 1). Attached to the rubber tube of the straight tube is a glass-funnel. The bottle is evacuated by the use of an ordinary suction-pump. Then a measured quantity of blood, *e.g.* 10 cc., is measured into the funnel; by opening the screw clamp below the funnel the blood is sucked into the bottle; after the blood $1\frac{1}{2}$ times its volume of distilled water are introduced in the same way and mixed with the blood by rotating; then through the funnel are added 4 cc. of fresh saturated solution of potassium ferricyanide and mixed with the contents of the flask by vigorous shaking; the flask is now placed in a bath at 40° and shaken at regular intervals during a period of about 30 minutes. There is no need to add ammonia to prevent the escape of CO_2 from the blood; on the contrary, the foaming which accompanies the liberation of CO_2 facilitates the decomposition of the CO-haemoglobin[1].

For the determination of the quantity of CO liberated after the extraction, outdoor air is introduced into the flask until atmospheric pressure is reached and the contents are thoroughly mixed; now one of the rubber tubes of one of the two bent tubes of the bottle is connected with the first Peligot tube (containing KOH) of the apparatus for CO determination, whilst the other is connected to a tube dipping into a jar filled with 2 litres of water. The last Peligot tube of the apparatus (*F*), containing potassium iodide, is connected to a suction pump, which is opened sufficiently to secure a passage of about 1 l. of gas through the apparatus in 30 minutes. When nearly all of the gas has passed, some amyl or capryl alcohol is introduced into the bottle to destroy the foam and the suction of the gas is continued until the bottle is full, when the screw clamp between the apparatus and the pump is closed and the bottle disconnected. The apparatus is now connected with another bottle containing outdoor air of which 1 or 2 l. is led through the apparatus to wash the I_2O_5. The KI solution from the last two Peligot tubes is now titrated, allowing for the iodine which is liberated in a blank determination made by passing 2 or 3 l. of outdoor air through the apparatus.

The usefulness of the technique described was tested in the following way. Fresh horse or ox blood was saturated with CO, and the content of CO determined as described. Then known quantities of this blood were mixed with fresh blood. The CO content of the resulting mixture was again determined and compared with the CO content as calculated. As at first the results were not satisfactory, it appeared that the fresh blood itself was not always CO-free, probably on account of the air in the stables. Table II indicates the results obtained.

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[1] This was found after most of the control experiments given in Table II had been accomplished; in them dilute ammonia was used.

Table II.

	cc. of CO in 10 cc. of blood		cc. of mixed blood		cc. of CO in 10 cc. of blood	
	Fresh	Saturated	Fresh	Saturated	Calculated	Found
1	0·009	1·10	70	3	0·054	0·058
2	0	0·72	90	10	0·072	0·095
3	0·062	1·01	95	5	0·109	0·112
4	0·051	1·46	95	5	0·122	0·121
5	0	1·26	90	10	0·126	0·126
6	0·034	1·49	90	10	0·180	0·200
7	0·069	1·32	90	10	0·194	0·215
8	Not determined	1·21	10	10	0·605	0·614
9	,,	1·29	10	10	0·647	0·685
10	,,	1·32	10	10	0·662	0·602
11	,,	1·29	0	10	1·29	1·23
12	,,	1·29	0	10	1·29	1·29
13	,,	1·32	0	10	1·32	1·32
14	,,	1·49	0	10	1·49	1·45

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XLVII. SOME REACTIONS OF RESTING BACTERIA IN RELATION TO ANAEROBIC GROWTH.

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I. Anaerobic growth of facultative anaerobes.

The circumstances in which the facultative anaerobes will grow under strictly anaerobic conditions are far from clear. It is known that the presence of carbohydrates capable of attack by the organism in question favours anaerobic growth; Ritter [1907] has found that, besides carbohydrates, some of the higher alcohols and hydroxy acids are efficient. Nitrates have been found to induce anaerobic growth of facultative anaerobes [Ritter, 1907; Veillon and Mazé, 1910] and Beijerinck [1903] has perceived the possibility that the liberation of energy from nitrate and sulphate oxidations might be utilised by micro-organisms for growth. A mass of data has accumulated on the bacterial reduction of nitrates to nitrites since the discovery of the phenomenon by Schönbein in 1868. Maassen [1901] has shown that 85 out of 109 types of bacteria tested will bring about the reduction. It is generally accepted to-day that the biological significance of the reduction of nitrate is that by it oxygen is supplied to an organism when free oxygen is no longer available.

The facts, however, are somewhat disconnected and little systematic analysis of the conditions under which anaerobic growth of the facultative anaerobes occurs appears to have been attempted.

II. The action of resting bacteria on nitrates.

Haas and Hill [1923] showed that in milk there was a system which could bring about the reduction of nitrate to nitrite and Dixon and Thurlow [1924] have been able to demonstrate that nitrate will replace methylene blue in the Schardinger reaction and in the xanthine oxidase reaction in milk.

There can be little question that nitrate is capable of acting as a hydrogen acceptor in a manner similar to methylene blue.

What part, then, is played by nitrate when this is present in a bacterial medium? Does it remove hydrogen in the sense that sulphite removes acetaldehyde in Neuberg's fermentations, and thus divert the course of the normal processes? Or does it play a more significant and positive rôle which would, for instance, be quite distinct from that of a fixative, or of methylene blue considered merely as a hydrogen acceptor?

Resting bacteria have been defined [Quastel and Whetham, 1924] as bacteria which have been grown for two days on tryptic broth, centrifuged, washed with saline and finally very well aerated. They are used under such conditions that multiplication of the cell does not occur. The reactions of the resting bacteria under our experimental conditions are thus due to the non-proliferating organism.

It was found that in the presence of resting bacteria succinic acid donates hydrogen to methylene blue with the production of fumaric acid. It was also found that in the presence of the bacteria fumaric acid oxidises leucomethylene blue, so that finally an equilibrium is established between succinic acid, fumaric acid, methylene blue and leucomethylene blue.

Now it is clear from the oxidising action of fumaric acid upon leucomethylene blue (which does not occur *in vitro*) that the resting bacteria activate the fumaric acid molecule. Activations of methylene blue and leucomethylene blue do not occur, for these are substances which are easily capable of accepting and donating hydrogen respectively *in vitro, i.e.* without activation. They form mixtures capable of giving definite oxidation-reduction potentials, measurable at an electrode, and are typical of normal oxidation-reduction systems of which the ferric-ferrous system is an example. Methylene blue and similar substances can therefore be regarded in a sense as already activated *in vitro*. Succinic acid, on the other hand, cannot donate hydrogen *in vitro*. Hence its power of reducing methylene blue *in vivo* must be ascribed to an activation of the acid by the cell. Similarly the oxidising action of fumaric acid *in vivo* must be attributed to an activation of this acid.

As in the case of muscle, there are many substances besides succinic acid which are donators of hydrogen to methylene blue in the presence of resting bacteria (*B. coli* or *B. pyocyaneus*). Among these are lactic acid, β-hydroxybutyric acid, glutamic acid, etc., and a detailed discussion of these will form the subject of a subsequent communication.

The influence of nitrate upon the rate of reduction of methylene blue by various hydrogen donators in presence of resting *B. coli* and resting *B. pyocyaneus* has been investigated. The experimental method was that employed in the work on succinic and fumaric acids [Quastel and Whetham, 1924].

Measurements of the velocity of reduction of methylene blue were made under anaerobic conditions in vacuum tubes at 45° in carefully buffered solutions at a p_{H} of 7·2.

The observations on methylene blue reductions recorded below are typical of the average of those made. The organism mainly employed for quantitative work was *B. coli comm.*, but *B. pyocyaneus* was sometimes used and found to give similar results.

The first experiments showed that nitrate has a most powerful inhibiting influence upon the velocity of reduction of methylene blue in presence of the bacteria. (See Table I.)

Table I.

Tubes	(1)	(2)	(3)
cc. of emulsion of resting *B. coli*	1	1	1
cc. phosphate buffer solution p_H 7·2	1	1	1
cc. 1 % Na succinate solution	0·5	0·5	–
cc. 1 % K nitrate solution	–	1	–
cc. methylene blue solution 1/5000	1	1	1
	Reduced completely in 8 mins.	Remained deep blue.	Not completely decolorised in 2 hrs.

It was expected that since nitrate can act as a hydrogen acceptor in a manner similar to methylene blue it would prolong the time of reduction of the dyestuff through competition for the hydrogen. Unless, however, there was a preferential attack of the nitrate, this would be no explanation for the complete inhibition of the reduction of methylene blue. This inhibition resembled that due to fumarates and for further analysis of the phenomenon recourse was had to the technique employed in demonstrating the oxidising action of fumaric acid *in vivo*.

Use is made of an inverted **U**-tube which can be evacuated. In one limb of the tube is placed a mixture of resting organism, buffer solution, hydrogen donator solution, and methylene blue, and in the other limb a solution of the substance whose oxidising action is to be investigated. The tube is evacuated and, if necessary, filled with nitrogen. The methylene blue is allowed to become completely reduced at 45°, and the solutions in the two tubes are mixed. If there occurs a permanent blueing it is evident that an oxygen donator is present. To show that the oxidation is due to a thermolabile enzyme, the tube containing the reduced methylene blue is heated to any desired temperature before the solutions are mixed. If after heating there is no permanent blueing it is concluded that a thermolabile enzyme controls the oxidation. Blank experiments must of course be made to show that the substance in question does not oxidise leucomethylene blue *in vitro*.

By making use of this technique the following facts were established:

(1) Nitrate oxidises leucomethylene blue in presence of resting *B. coli* or *B. pyocyaneus*, but not *in vitro* (at p_H 7·2 and 45°). In equivalent concentration it is more powerful than fumarate in oxidising the leuco dyestuff.

(2) The oxidation by nitrate is dependent, like that by fumaric acid, on a thermolabile enzyme.

In one limb of a vacuum **U**-tube was placed a mixture of 1 cc. resting *B. coli*, 1 cc. 1 % sodium succinate solution, 1 cc. phosphate buffer solution, p_H 7·2, and 1 cc. 1/5000 methylene blue solution, and in the other 1 cc. of a 5 % sodium nitrate solution.

The technique was carried out as described above and no oxidation of the leucomethylene blue was found to occur; if the organism had not been heated the oxidation would have been extremely rapid.

(3) Nitrite cannot reduce methylene blue or oxidise leucomethylene blue in presence of resting *B. coli* or *in vitro* (at p_H 7·2 and 45°).

The enzyme dealing with nitrate, like that which deals with succinic acid and fumaric acid, appears to be closely associated with the physical structure of the organism. It is destroyed when the organism becomes coagulated either by heat or by treatment with acid.

These results suggest that when nitrate is added to a nutrient medium containing *B. coli* or *B. pyocyaneus* the nitrate around the cells becomes activated so that it is capable of oxidising substances in the vicinity of the cell (or at the cell surface) which it is incapable of oxidising *in vitro* (or in the body of the medium where the nitrate is outside the sphere of influence of the cell). Now there must be a definite pressure of nitrate molecules on every cell in the medium and since a number of these molecules become activated at the cell surface, each organism will become the centre of a sphere where active oxidations will proceed. Each cell may then be regarded as in a state comparable with that which exists under aerobic conditions.

In this manner the action of nitrate offers a distinct contrast to that of methylene blue, which does not require activation by the organism. Moreover, since the cell surface, like any enzyme, will become "saturated" with regard to the activated nitrate, the oxidation conditions around the cells, above a certain concentration of nitrate, will remain constant. Since nitrite is inert from the point of view of oxidation and reduction (*i.e.* by *B. coli*) and is an easily diffusible ion, its production will not affect the oxidation conditions, and hence the velocity of oxidation of a hydrogen donator at the cell surface. With methylene blue, however, the oxidation potential at the cell surface will depend on the relative proportion of methylene blue to leucomethylene blue and will vary as this proportion alters.

III. The action of resting bacteria on chlorates and perchlorates.

Using the same technique as with nitrate it was found that chlorate is a most powerful (anaerobic) oxygen donator. It will not oxidise leucomethylene blue *in vitro* but a vigorous oxidation occurs in the presence of resting *B. coli* or *B. pyocyaneus*. In the presence of chlorates complete reduction of methylene blue by a hydrogen donator *in vivo* does not appear to take place. Chlorate, moreover, will not effect the reduction of methylene blue by cysteine or glutathione (*i.e. in vitro* reduction); it will do so, however, in the presence of resting organism. As in the case of fumarates and nitrates, the action of chlorates depends on a thermolabile enzyme. (See Table II.)

Perchlorates neither oxidise leucomethylene blue *in vivo* nor *in vitro*. Bromates and iodates both oxidise leucomethylene blue *in vitro*.

IV. The action of resting *B. subtilis* on nitrates and chlorates.

Resting *B. subtilis* was investigated in a similar manner to *B. coli* and *B. pyocyaneus* and it was found that neither nitrates nor chlorates bring about any perceptible oxidation of leucomethylene blue in presence of the organism. Fumarate oxidises leucomethylene blue in the presence of the organism only very slowly and certain strains do not appear to produce any oxidation. If *B. subtilis* can activate fumarate, nitrate or chlorate at all, the activation is very slight indeed.

Table II.

Tubes	(1)	(2)	(3)
cc. 1 % Na chlorate solution	1	1	1
cc. buffer solution p_H 7·2	1	1	1
cc. methylene blue solution 1/5000	1	1	1
cc. cysteine solution 4 mg./cc.	1	1	1
cc. resting *B. coli*	1	–	–
cc. resting *B. coli* which has been heated at 100° for 20 mins.	–	1	–
	Reduction of methylene blue never complete.	Rapid reduction in 10 mins.	Rapid reduction in 10 mins.

It is well known, of course, that *B. subtilis* will not bring about the reduction of nitrate to nitrite in a fermenting medium, whilst *B. coli* and *B. pyocyaneus* do so very readily.

V. CONSIDERATION OF SOME CONDITIONS NECESSARY FOR GROWTH.

It is clear that for an organism to grow energy must be supplied to it for its synthetic operations. This is secured either by the oxidation of the substrate or by its anaerobic breakdown (occurring with the liberation of energy). It has been suggested [Stephenson and Whetham, 1924] that in the anaerobic growth of *B. coli* upon glucose the energy is derived from the anaerobic breakdown of glucose into lactic acid, a change involving the liberation of 22·56 Cal. per gram-mol. of glucose. It was shown, in fact, that even in the presence of air the early period of the growth of *B. coli* upon a glucose medium is anaerobic.

With lactic acid, succinic acid, glycerol, etc., all the likely paths of anaerobic breakdown appear to involve absorption of energy and on none of these compounds, where these formed the sole sources of carbon, and ammonia the sole source of nitrogen, was anaerobic growth of *B. coli* found to occur.

Beijerinck and Folpmers [1916] showed that fumaric acid and malic acid can be broken down by bacteria to pyruvic acid, and one of us [Quastel, 1924] was able to demonstrate that in the growth of *B. pyocyaneus* on ammonium fumarate, the whole of the oxygen absorbed during the first two or three days' growth was accounted for by oxidation of the fumaric acid to oxalacetic (or pyruvic) acid. Within the limits of experimental error no oxygen uptake by the bacteria alone could be demonstrated. In other words the molecular oxygen utilised simply oxidised the fumaric acid to pyruvic acid. The latter molecule entered into the organism's synthetic operations and the necessary energy for synthesis was derived from the fumaric acid oxidation. This point is worthy of emphasis for it clearly demonstrates that given (1) an adequate source of energy, (2) the appropriate molecule to enter into the organism's synthetic reaction, the presence of molecular oxygen may not be necessary for growth.

There is another point to consider. If the organism is incapable of activating a substrate molecule, *i.e.* if it does not possess a mechanism which will induce the molecule to liberate energy (*e.g.* either by oxidation or by anaerobic breakdown), it is clear that this molecule cannot serve as a basis for a nutrient medium. For instance, if an organism cannot activate fumaric acid so that the latter may become oxidised so as to liberate energy and form pyruvic acid, this organism will not grow upon ammonium fumarate as sole source of carbon and nitrogen.

B. subtilis cannot activate fumaric acid to any appreciable extent (see above) and, as has been previously shown, it does not grow aerobically (or anaerobically) on ammonium fumarate. *B. coli* and *B. pyocyaneus* activate fumaric acid and both grow aerobically upon ammonium fumarate.

The following three conditions at least seem, therefore, to be necessary before growth may occur:

(1) The organism must be able to secure energy either by anaerobic decomposition of the substrate (*e.g.* glucose into lactic acid) or by the oxidation of the substrate.

(2) The organism must be able to activate the substrate so that the latter is capable of reaction, *e.g.* oxidation or reduction, which it does not necessarily undergo *in vitro*.

(3) The products of oxidation (or decomposition) of the substrate, or the substrate itself, must be capable of entering into the synthetic operations of the organism.

It follows that if molecular oxygen is replaced by some molecule which induces around the bacterial cell a state comparable with that which occurs under aerobic conditions, and so long as the three conditions enumerated above are fulfilled, then anaerobic growth should be possible in presence of this molecule.

It has been shown above that we have reason to suppose that nitrate produces about the cell a state comparable with that existing under aerobic conditions. Chlorate and fumarate, which both oxidise leucomethylene blue in presence of resting *B. coli* and *B. pyocyaneus* should therefore have effects similar to those of nitrate. Provided, then, that conditions (1), (2) and (3) enumerated above are complied with, the presence of one of these substances (nitrate, chlorate or fumarate) should enable *B. coli* or *B. pyocyaneus* to grow anaerobically on compounds, such as lactic acid, which will not alone normally support anaerobic growth.

Condition (1) is satisfied by lactate and nitrate, and lactate and fumarate, as the following equations show:

$$KNO_3 + CH_3 \cdot CHOH \cdot COOH = KNO_2 + CH_3 \cdot CO \cdot COOH + H_2O + 30{\cdot}7 \text{ Cals.}$$

$$COOH \cdot CH{=}CH \cdot COOH + CH_3 \cdot CHOH \cdot COOH = COOH \cdot CH_2 \cdot CH_2 \cdot COOH + CH_3 \cdot CO \cdot COOH + 16 \text{ Cals.}$$

Condition (2) is satisfied as shown by the results described in section II.

Condition (3) is satisfied by the production of pyruvic acid.

Since perchlorate on the other hand is not activated by *B. coli* or by *B. pyocyaneus*, it cannot be expected to produce anaerobic growth or oxidation, and since nitrate is not apparently activated by *B. subtilis* it should not induce the anaerobic growth of this organism.

VI. ANAEROBIC GROWTH IN THE PRESENCE OF NITRATES.

Throughout the following experiments the inorganic medium employed was as follows:

0·4 g. $(NH_4)_2HPO_4$.
0·1 g. NaCl.
0·1 g. KH_2PO_4.
0·07 g. $MgSO_2 7H_2O$.
100 cc. distilled water.

To this was added 1% of a carbon compound.

The solution was brought to a p_H of 7·2 and autoclaved in test-tubes in 10 cc. lots. Each of them was inoculated with 0·1 cc. of a 20-hour broth culture of *B. coli* or *B. pyocyaneus* and incubated aerobically or anaerobically as required. Experience has shown that it is very desirable to inoculate with relatively heavy sowings of the bacteria in order to obtain consistent and regular results.

The results are enumerated below.

A. with *B. coli*.

(1) Growth of *B. coli* occurs under strictly anaerobic conditions in the presence of nitrates, and with pyruvic acid, lactic acid, succinic acid, fumaric acid or glycerol as the sole source of carbon. No growth occurs anaerobically on these substances in the absence of nitrates.

(2) *B. coli* produces from lactic acid aerobically and anaerobically in the presence of nitrates a relatively large quantity of pyruvic acid. The yield is of the order obtained from *B. pyocyaneus* growing upon fumaric acid. *B. coli* when growing aerobically upon lactic acid in the absence of nitrates does not liberate pyruvic acid. This phenomenon will be discussed in more detail later.

(3) Growth of *B. coli* in the presence of nitrates is always accompanied by the production of large quantities of nitrite.

B. with *B. pyocyaneus*.

(1) Extensive growth of *B. pyocyaneus* occurs in the presence of nitrates under strictly anaerobic conditions on citric acid, glucose, lactic acid, or pyruvic acid as sole source of carbon. *B. pyocyaneus* will not grow anaerobically upon these substances (with the exception of glucose) in the absence of nitrates. It will grow anaerobically to some extent on glucose alone without nitrates, but not nearly to the same extent as with nitrates. *B. coli*, on the other hand, grows very well on glucose alone anaerobically and the growth is similar to that obtained when nitrates are present.

B. pyocyaneus both aerobically and anaerobically liberates pyruvic acid from lactic acid in the presence of nitrates. It will produce pyruvic acid from lactic acid aerobically in the absence of nitrates (difference from *B. coli*). The growth of *B. pyocyaneus* upon citric acid and nitrate anaerobically and upon citrate alone aerobically is extremely luxuriant. We have found it very convenient to use a tube of citrate medium (containing no nitrate) inoculated with *B. pyocyaneus* as a test for anaerobiosis. In the presence of a trace of oxygen growth will occur.

Good growth, but not so extensive as with citric acid, occurs anaerobically in the presence of nitrates with succinic acid, fumaric acid, or glycerol as sole source of carbon.

(2) *B. pyocyaneus* breaks down nitrites anaerobically. This is not the case with *B. coli*. (This was originally observed by Maassen [1901].)

C. with *B. subtilis*.

No anaerobic growth of this organism has yet been obtained. This agrees with the observation that *B. subtilis* is incapable of activating nitrate.

It is interesting to note that *B. coli* which will not produce pyruvic acid from lactic acid aerobically in the absence of nitrates, will do so in the presence of nitrates. It has been shown by Mazé [1918] that pyruvic acid is formed from lactic acid by several micro-organisms and we have noted the excellent yields of pyruvic acid produced by *B. pyocyaneus* in a well-aerated medium. In the work on fumaric acid it was shown that *B. pyocyaneus*, but not *B. coli*, will liberate pyruvic acid. It was questioned whether this difference between the two organisms was due to the absence from *B. coli* of a specific enzyme present in *B. pyocyaneus*. Later evidence was brought to show [Quastel and Whetham, 1924] that "the non-appearance of a ketonic acid when *B. coli* is grown on a fumarate medium might be explained as due to differences between the rates of oxidation by growing *B. coli* and growing *B. pyocyaneus* and not to the absence from the former of a specific enzyme capable of producing the ketonic acid." This view is clearly corroborated by the nitrate experiments described above. It also agrees with Mazé's observations on the production of pyruvic acid from lactic acid.

VII. THE EFFECT OF RESTING *B. COLI* ON LACTATE AND NITRATE: ISOLATION OF PYRUVIC ACID.

20 cc. of a thick suspension of resting *B. coli* were placed in a vacuum tube capable of holding 100 cc. To this were added 10 cc. of a neutral 1 % sodium lactate solution, 10 cc. of a 5 % sodium nitrate solution and 10 cc. of a phosphate buffer solution, p_H 7·2. The tube was evacuated and placed in a thermostat at 45° for three hours. The contents of the tube were then centrifuged and the clear supernatant solution treated with phosphoric acid and warmed. Urea was added to remove all nitrite. After cooling, a freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid was added, and the *p*-nitrophenylhydrazone of pyruvic acid was separated and identified.

VIII. THE EFFECT OF RESTING *B. COLI* ON LACTATE AND CHLORATE: PRODUCTION OF CHLORITE AND PYRUVIC ACID.

The same procedure was adopted as in the above experiment, using chlorate solution instead of nitrate.

After three hours at 45° the presence of pyruvic acid could be demonstrated by the nitroprusside reaction. It was isolated as the *p*-nitrophenylhydrazone. Some chlorate had been reduced to chlorite. The presence of chlorite could be demonstrated by adding a little potassium iodide to the solution, a little starch solution and finally dilute acetic acid. There was an immediate liberation of iodine. Hypochlorite was not formed since this would have reacted with potassium iodide in neutral or alkaline solutions. The chlorite can be estimated iodometrically in the presence of dilute acetic acid.

The oxidising effect of chlorate upon lactate in presence of resting *B. coli* is more vigorous than that of nitrate for equivalent concentrations. No oxidation of lactate by either nitrate or chlorate occurs *in vitro*.

IX. THE COURSE OF THE PRODUCTION OF NITRITE AND PYRUVIC ACID DURING THE GROWTH OF *B. COLI* ON LACTATE AND NITRATE.

B. coli was grown aerobically and anaerobically in an inorganic medium containing 1 % lactic acid as sodium lactate, and 1 % sodium nitrate, and the production of nitrite and pyruvic acid was followed in both conditions.

The tubes were inoculated as usual and incubated aerobically and anaerobically. They were examined in duplicate after varying intervals of time. The nitrite produced was estimated iodometrically. 2 cc. of the medium were placed in a small wash bottle, a little potassium iodide and starch solution were added and nitrogen bubbled through vigorously. Dilute sulphuric acid was added and the iodine liberated was estimated by titration with $N/10$ thiosulphate solution.

The pyruvic acid production was followed by the nitroprusside reaction. To 2 cc. of the medium were added solid ammonium sulphate, a few crystals of sodium nitroprusside and finally 2 cc. of strong ammonia. In the tables below a single plus sign indicates a positive but feeble reaction, whilst a number of plus signs indicates an intense reaction demonstrating the production of a large quantity of pyruvic acid. Table III gives the results of one aerobic series and Table IV gives the results of a parallel aerobic and anaerobic series. The maximum amount of sodium nitrite possible from 1 % sodium nitrate is 0·81 %.

It is generally found that the pyruvic acid liberation by *B. coli* growing in a medium containing 1 % lactate and 1 % nitrate is irregular and feeble until the nitrite produced reaches a concentration equivalent approximately to 0·4 % sodium nitrite. After this concentration is reached the pyruvic acid liberation is increased enormously, whilst the actual amount of growth does not appear to increase.

Table III.

Hours from time of inoculation	Growth	Pyruvic acid produced (examined by nitroprusside reaction)	p_H	% sodium nitrite produced
15	+ + +	+ +	6·8	0·45
20¾	+ + +	+ + + +	6·8	0·57
31¾	+ + +	+ + + + +	7·1	0·65
41½	+ + +	+ + + + +	7·25	0·76
48	+ + +	+ + + + + (0·3 % by colorimetric comparison)	7·3	0·79
65*	+ + +	+ + + + +	7·4	0·81

* An examination of these tubes showed that practically all the cells present were dead.

Table IV.

Hours from time of inoculation	Growth		Pyruvic acid (indicated by nitroprusside reaction)		% sodium nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
18	+ +	+ +	+	+	0·33	0·34
23	+ + +	+ + +	+	+	0·38	0·38
41½	+ + +	+ + +	+ + + + +	+ + + + +	0·69	0·72
66	+ + +	+ + +	+ + + + +	+ + + + +	0·76	0·77

Moreover this course of events occurs both in aerobic and anaerobic conditions. In other words *B. coli* grows on a mixture of lactate and nitrate in the same way whether the conditions are aerobic or anaerobic.

Pyruvic acid was isolated after approximately 50 hours' growth in the following way. To each tube a little chalk was added and the mixture well shaken and filtered. The filtrate, which was practically free from protein matter, was treated with syrupy phosphoric acid (1 cc. of acid for each tube), excess urea was added, the solution warmed and finally well aerated. When all the nitrite was decomposed, the solution was refiltered, cooled, and treated with a little freshly-prepared saturated solution of *p*-nitrophenylhydrazine in glacial acetic acid. The precipitated hydrazone was filtered through a Buchner funnel, washed with glacial acetic acid and water and recrystallised from dilute alcohol.

It was identified as the *p*-nitrophenylhydrazone of pyruvic acid by its melting point (219°) and mixed melting point.

X. THE TOXIC EFFECT OF NITRITE UPON *B. COLI*.

To a series of tubes containing 1 % lactate and 1 % nitrate was added sodium nitrite in varying quantities. These tubes were inoculated with *B. coli* and the growth and amount of nitrite present after 16 hours' incubation were examined in the usual way. The following table gives the results.

Table V.

% nitrite present initially	Growth after 16 hours		% nitrite present after 16 hours		% nitrite produced	
	Aerobic	Anaerobic	Aerobic	Anaerobic	Aerobic	Anaerobic
0	+ + +	+ + +	0·45	0·45	0·45	0·45
0·01	+ + +	+ + +	0·30	0·32	0·29	0·31
0·05	+ +	+ +	0·27	0·28	0·22	0·23
0·15	+	+	0·20	0·19	0·05	0·04
0·35	0	0	0·36	0·36	0·01	0·01
0·6	0	0	0·6	0·6	0·0	0·0

The concentration of sodium nitrite toxic to *B. coli* in a 1 % lactate 1 % nitrate medium appears to be about 0·35 %. Although this strength of nitrite causes cessation of growth of *B. coli* it does not prevent resting *B. coli* from activating nitrate and lactate, so that the latter interact and produce nitrite and pyruvic acid. This is illustrated by the following experiment. A series of tubes containing 1 % lactate, 1 % nitrate and 0·4 % nitrite was prepared. To each tube was added 1 cc. of resting *B. coli*. After 20 hours each tube showed an increase in the quantity of nitrite present and pyruvic acid was formed as indicated by the positive nitroprusside reaction. This phenomenon affords an explanation of the fact that much pyruvic acid does not appear during the growth of *B. coli* on a lactate-nitrate medium until the nitrite production has reached about 0·4 %. Below this strength of nitrite the pyruvic acid produced from the lactate is almost entirely used for growth. As soon, however, as the nitrite reaches its toxic concentration and prevents further bacterial synthesis, the pyruvic acid, which is still being produced from the lactic acid by the action of nitrate and the resting organism, is diverted from synthesis and is liberated into the medium.

XI. The breakdown of nitrite by *B. pyocyaneus*.

The breakdown of nitrite by *B. pyocyaneus* is a well-known phenomenon. The following experiment shows the ease with which it is effected.

A medium containing 1 % lactate and 0·1 % nitrite and another containing 1 % lactate and 0·05 % nitrite were sterilised and sown with 0·1 cc. of a 20-hour broth culture of *B. pyocyaneus* and incubated aerobically. The following table gives the results.

Table VI.

Hours after time of inoculation	0·05 % nitrite (as K salt) medium	0·1 % nitrite (as K salt) medium
21½	All nitrite utilised	0·03 % nitrite still present
41½	—	All nitrite utilised

XII. Aerobic and anaerobic growth of *B. coli* in the presence of chlorates.

So far it has been found impossible to substitute chlorates for nitrates in anaerobic growth, although the organism has been shown to be capable of activating chlorate. This failure of chlorate to induce anaerobic growth is probably due to the production of chlorite (p. 312), which is toxic to *B. coli* at a concentration of 1 in 20,000.

The following series of experiments (see Table VII) shows the toxic effect of chlorate on *B. coli* growing on lactate and nitrate.

Tubes containing 1 % lactate, 1 % nitrate and varying quantities of potassium chlorate were inoculated in the usual way and incubated at 37° both aerobically and anaerobically.

Table VII.

% $KClO_3$	0·5	0·1	0·025	0·005	0·001	0
Aerobic.						
Growth after 16 hours from time of inoculation	0	0	? +	+ + +	+ + +	+ + +
Growth after 44 hours from time of inoculation	+	+	+	+ + +	+ + +	+ + +
Pyruvic acid production (indicated by nitroprusside reaction)	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + +
Anaerobic.						
Growth after 44 hours from time of inoculation	0	0	+	+ + +	+ + +	+ + +
Pyruvic acid production (indicated by nitroprusside reaction)	0	0	0	+ + + +	+ + + +	+ + +

The concentration of potassium chlorate, which is completely toxic to *B. coli* anaerobically under these conditions, is about 0·03 % or 1 in 3000. Aerobically there is a definitely toxic effect of chlorate at about this concentration, but there is slight growth at concentrations of chlorate much higher than 0·03 %.

XIII. The toxic effect of chlorites.

Potassium chlorite was prepared according to the method of Bruni and Levi [1915]. The chlorite obtained was estimated iodometrically (in the presence of dilute acetic acid) and the solution made up with distilled water to 1 %. It was brought to p_H 7·2. The solution was not autoclaved since considerable loss of chlorite is occasioned by so doing—moreover, it is unnecessary since chlorite is a most powerful antiseptic.

Tubes containing 1 % lactate 1 % nitrate medium were prepared and sterilised; to these were added varying quantities of chlorite and the tubes were inoculated with *B. coli*. They were incubated aerobically and anaerobically. Table VIII gives the results.

Table VIII.

% $KClO_2$	0·07	0·05	0·03	0·01	0·005	0
Aerobic.						
Growth after 24 hours	0	0	0	0	(+)	+ + +
" " 48 "	0	0	0	0	(+)	+ + +
Anaerobic.						
Growth after 48 hours	0	0	0	0	(+)	+ + +

The fact that resting *B. coli* has been shown to produce chlorite from chlorate and the fact that chlorite is toxic at extremely low concentrations explains why chlorate is ineffective as a substitute for nitrate in anaerobic growth.

XIV. AEROBIC AND ANAEROBIC GROWTH OF *B. COLI* IN THE PRESENCE OF PERCHLORATES AND OF MALEIC ACID.

Since perchlorate and maleate [Quastel and Whetham, 1924] do not appear to be activated by resting *B. coli* it was expected (1) that perchlorates and maleates would not act as substitutes for nitrates in anaerobic growth; (2) that perchlorates and maleates would not be toxic to *B. coli* growing upon lactate and nitrate.

Experiment shows that neither perchlorates nor maleates will induce anaerobic growth and that they have no perceptible toxic action on *B. coli* growing upon lactate and nitrate, under aerobic or anaerobic conditions.

XV. AEROBIC AND ANAEROBIC GROWTH IN THE PRESENCE OF FUMARATE.

If the general conceptions which have been outlined above are correct it should follow that for anaerobic growth nitrates may be replaced by some organic hydrogen acceptor or oxygen donator, so long as this substance is activated by the organism and there is the necessary liberation of energy, etc.

Fumaric acid was chosen as a suitable hydrogen acceptor and experiment showed that *B. coli* will grow anaerobically (in the absence of nitrates) on a mixture of lactate and fumarate where these substances form the sole sources of carbon and ammonia the sole source of nitrogen. On lactate alone and on fumarate alone no anaerobic growth of *B. coli* occurs. For experiment the following solutions were made up:

A. 1 % lactic acid as Na salt, 1 % fumaric acid as Na salt, and the usual inorganic medium.

B. The same with 2 % fumaric acid.

These were autoclaved, inoculated as usual, and incubated aerobically and anaerobically. The results are indicated in Table IX.

Table IX.

	1 % lactate, 1 % fumarate medium	1 % lactate, 2 % fumarate medium
Aerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	7·0
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	7·6	7·0
Anaerobic.		
Growth after 48 hours	—	Very good throughout medium
p_H after 48 hours	—	6·8
Growth after 72 hours	Very good throughout medium	Very good throughout medium
p_H after 72 hours	6·8	6·8

When *B. coli* grows aerobically on fumarate or anaerobically on a mixture of fumarate and nitrate the solution becomes alkaline ($p_H > 7{\cdot}4$) owing to the decarboxylation of the oxidised fumaric acid. Since lactic acid is a monobasic acid the growth of *B. coli* upon this does not produce immediate alkalinity, but a slight acidity owing to the ammonia which is utilised being more than equivalent to the alkali set free by the utilisation of the lactic acid. Hence, if in a mixture of lactic and fumaric acids there is produced immediate alkalinity after growth of *B. coli*, this would show that fumaric acid is being utilised; if an acidity, this would be evidence for the utilisation of lactic and the non-utilisation of fumaric acid. In all the anaerobic growths of *B. coli* on mixtures of lactic and fumaric acids there is produced slight acidity indicating the utilisation of lactic acid and that the fumaric acid is acting solely as hydrogen acceptor. This was further confirmed by the isolation of succinic acid from these media.

For experiment, twenty tubes, each containing 10 cc. of a 1 % lactate, 1·25 % fumarate medium, were inoculated with *B. coli* and incubated anaerobically for 12 days. All tubes showed good growth; the p_H of the solution was 6·9. The contents of the tubes were mixed and 20 cc. of syrupy phosphoric acid were added. The acid solution was extracted with ether and the white residue from the ether extract dissolved in a little water, silver nitrate solution added, the mixture filtered, and the clear filtrate just neutralised with sodium hydroxide. There was a copious precipitate, which, on decomposition by sulphuretted hydrogen, yielded a solution which did not reduce permanganate and gave a crystalline residue of succinic acid, identified by the melting point (185°) and by a mixed melting point.

It is specially noteworthy that fumaric acid can play a dual rôle:

(1) It will act as an oxygen acceptor and become a nutrient carbon source, *e.g.* in the anaerobic growth of *B. coli* on fumarate and nitrate.

(2) It will act as a hydrogen acceptor and so allow lactic acid (for instance) to be utilised, *e.g.* in the anaerobic growth of *B. coli* on a mixture of fumarate and lactate.

We wish to express our appreciation of the continued interest of Sir F. G. Hopkins in this work. Two of us (M. S. and M. D. W.) have been enabled to pursue these investigations through grants received from the Medical Research Council.

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XLVIII. THE INFLUENCE OF BLOOD SERUM AND OF SUGARS ON HAEMOLYSIS.

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Of late considerable interest has been shown in the phenomenon of inhibition of haemolysis, a matter of no little importance. The experiments of Ransom [1901] have been widely quoted, and passed from book to book, as though worthy of complete reliance. His conclusion that the inhibition of saponin haemolysis is due to the cholesterol content of the serum is negatived, however, by the fact that the quantity of cholesterol in serum is not sufficient to bring about the effects. Stocks [1919] more recently investigated the question, and concurs with Ransom, but his results are directly at variance with those of Ponder [1923], who has shown that the protein content of the serum is the chief factor in the production of inhibition. Stocks also believes that the inhibition is the result of a simple chemical reaction, but it can easily be shown that the inhibitory process is a much more complicated one than he would make out. It has therefore been thought well to confirm the findings of Ponder, who has investigated only the action of serum and of simple proteins, and to find out whether other inhibitory substances conform to the particular relation described by him.

Method.

The method adopted is the neat and simple one devised by Ponder, whose papers may be consulted [1921, 1922].

The materials used require some note. The haemolysin is pure white saponin dissolved in 0·85 % NaCl. The cell suspension is one of human cells, prepared by drawing off 1 cc. of blood into citrated saline, washing the cells three times, and then suspending them in 20 cc. of 0·85 % NaCl. A fresh suspension is made up every day. The sera employed were obtained from the clotted blood of the animal concerned, and were used fresh. The sugar solutions were made from pure sugars, dissolved in distilled water, and were isotonic with the cells.

Technique.

The technique of Ponder was closely followed, the result being the production of two curves for each sugar, one showing the relation between the time for the production of complete haemolysis and the dilution of haemolytic agent in the absence of any inhibitory substance, and the other the relation in the presence of the sugar or serum. The logarithm of the difference between the δ coordinates of the two curves at any time t, is then plotted against the logarithm of the δ coordinate of the lower of the two curves at time t, the dilutions being first converted into concentrations. The result of such a plotting is a straight line as shown in Fig. 2. A typical experiment is set out in the following table; δ_1 is the dilution of lysin employed, c_1 its corresponding concentration, T the time for haemolysis, and δ_2 the dilution of lysin which is considered as acting on the cells and not inhibited; c_2 is its corresponding concentration. From these figures the curves shown in Figs. 1 and 2 are derived. In this case glucose is the inhibitory substance.

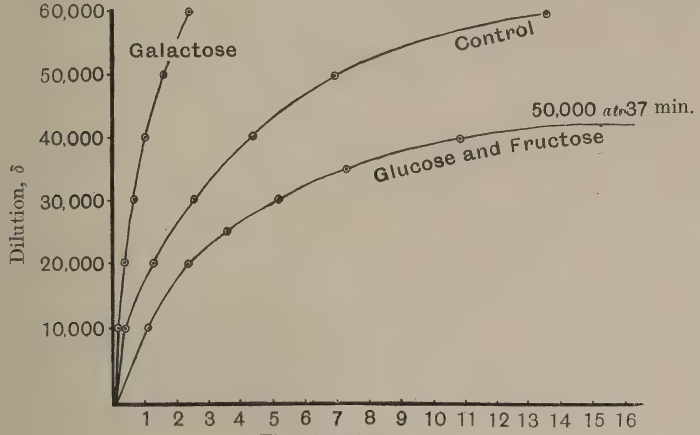

Fig. 1

δ_1 1 in	c_1 mg.	T min.	δ_2 1 in	c_2 mg.	x mg.	log c_2	log x
10,000	0·2	1·0	18,000	0·11	0·09	$\bar{1}{\cdot}04$	$\bar{2}{\cdot}95$
20,000	0·1	2·4	30,000	$0{\cdot}0\dot{6}$	$0{\cdot}0\dot{3}$	$\bar{2}{\cdot}82$	$\bar{2}{\cdot}52$
25,000	0·08	3·5	36,000	0·055	0·027	$\bar{2}{\cdot}74$	$\bar{2}{\cdot}43$
30,000	$0{\cdot}0\dot{6}$	5·1	43,000	0·046	0·02	$\bar{2}{\cdot}66$	$\bar{2}{\cdot}30$
35,000	0·057	7·2	50,000	0·04	0·017	$\bar{2}{\cdot}60$	$\bar{2}{\cdot}23$
40,000	0·05	10·7	57,000	0·035	0·015	$\bar{2}{\cdot}54$	$\bar{2}{\cdot}17$

Results.

(1) *Serum.* For these experiments rabbit serum was used in a dilution such that 0·01 cc. was present in each tube with the saponin. The following results were obtained:

Animal	Value of n	Value of A
1	0·93	1·2
2	0·93	1·09
3	1·0	1·0
4	1·3	0·4
5	0·9	1·12

Ponder gives only one case of the rabbit, in which the constants are $n = 1{\cdot}125$, $A = 1{\cdot}02$; it will be seen that all the above results give considerably lower values of n than the figures for the sera of the cat and of man, so that, for identical dilution, rabbit serum appears to be less inhibitory. The errors of these values are exceedingly slight, and no difficulty was found in any case in reducing them to the linear form.

(2) *Glucose and Fructose.* Very pure samples were used, and the solutions made up fresh every time they were required. No effect on the cells was observed as a result of the sugars *per se*. It was found that glucose and fructose acted identically when in the same concentration, the curves being so nearly coincident that the one given by fructose has been omitted from the graph in Fig. 1. It will be seen that the effect of these sugars on saponin haemolysis is substantially the same as that of serum, the linear form of graph being obtained by plotting log x against log c_2. The values of the constants are $n = 0{\cdot}77$, $A = 1{\cdot}0$. This value of n is much less than is usually found in serum experiments.

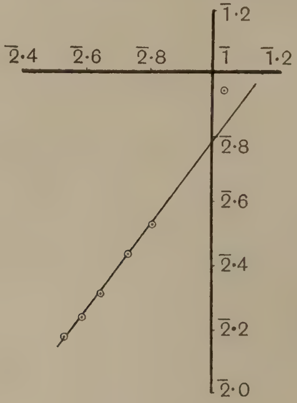

Fig. 2

Note. The uppermost point lies somewhat off the line, but this divergence is really insignificant, and is occasioned by its greater liability to experimental error, and to the sensitiveness of logarithms in this region.

(3) *Galactose.* A very different effect was here observed. The action is to accelerate in a marked manner the haemolytic rate, as can be seen from the uppermost curve in Fig. 1, although the sugar has no effect on the cells *per se*. So far no method of analysing the acceleration curve has been devised.

Careful experiments were conducted to discover whether the acceleration effect was in any way comparable to the accelerations met with in systems of bile salts and serum, or bile salts and histamine. In such systems the histamine or serum acts as an inhibitor if added to the cell suspension before the glycocholate, and as an accelerator if added after the cells are mixed with the lysin [Ponder, 1922]. This was not found to be so in the galactose-saponin system, nor indeed, was it expected to occur, since no such inhibition-acceleration effects have been observed with saponin, which shows either a clear inhibition as with serum, or a clear acceleration as with aspartic acid and allied substances.

(4) *Starch.* Further experiments were carried out with a highly purified form of rice starch, in many dilutions, but no effect on the saponin haemolysis was observed.

It is not proposed to discuss these results, which are in part confirmatory ones, nor can the reason for the acceleration produced by galactose be dealt with. It is however interesting to note that two such closely allied sugars as glucose and galactose should have such diametrically opposed effects on the action of saponin.

SUMMARY.

1. Certain results are recorded confirming Ponder's results on the inhibition of saponin haemolysis by serum.

2. Glucose and fructose inhibit saponin haemolysis in a manner much like that of serum.

3. Galactose accelerates the action of saponin, and starch has no effect thereon.

This research was carried out during the tenure of a Carnegie Scholarship.

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XLIX. THE RELATION BETWEEN SULPHUR AND NITROGEN METABOLISM.

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(Received January 12th, 1925.)

The purpose of the present paper was an attempt to elucidate the following controversial points in protein metabolism.

1. Is the breakdown of ingested protein immediate or is it spread over several days?
2. Is there any difference in the rates at which nitrogen and sulphur are excreted?
3. If ingested protein can be retained is it capable of covering the breakdown of body protein in the first few days of starvation?
4. What is the nature of the stored material?

Is ingested protein dealt with immediately? This question was repeatedly investigated by the older workers, but it was not until the introduction of the superimposition method of Falta that clear proof was obtained that a real delay in excretion may take place. Falta [1906] gave a subject a constant diet until nitrogen equilibrium was established and then on one day superimposed on this diet a known amount of the protein to be investigated. The course of its excretion was then followed for several days. The amount of the protein excreted in each particular day was found by subtracting the total nitrogen output on the basal diet from the nitrogen output on the day concerned. He found that the time of excretion varied with different proteins. Hämäläinen and Helme [1907], using this method, found the same delay in the metabolism. The general conclusion is that there is a retention in some form of the nitrogen of the food.

Is there any difference in the rate at which the nitrogen and sulphur of the ingested protein is excreted? Feder [1881], by following the outputs of nitrogen and sulphur in 2-hourly samples of the urine of a dog after meat ingestion, found an early excretion of sulphur and a delay in the nitrogen output. He also emphasised the importance of 2-hourly samples as opposed to the 24-hourly collection. Falta [1906] found, taking the 24-hour urine, an early excretion of sulphur after the ingestion of egg white and caseinogen. Hämäläinen and Helme [1907] obtained the same result with egg white, veal and proton (caseinogen). Wolf and Osterberg [1911] maintained that when egg white, either raw or coagulated, was superimposed sulphur was more slowly excreted than nitrogen in the early hours. Lewis [1916] found a lag in the sulphur output as compared with the nitrogen on feeding meat to dogs after starvation. Cathcart and Green [1913], on the other hand, observed a nitrogen lag after the ingestion of gelatin and egg white. The evidence then as regards the rate of excretion of sulphur and nitrogen is on the whole rather contradictory. In regard to the nature of the material stored there has been a certain amount of inconclusive work. Falta thought the variation in the rate of excretion of the nitrogen and sulphur after the ingestion of different proteins was due to the differences in their resistance to breakdown. Ehström [1906], on the other hand, considered that the material was stored in some easily metabolisable form. Obviously, however, if there are differences in the rate at which nitrogen and sulphur are excreted then the stored material must differ in composition from the protein ingested. In the present experiments an attempt has been made to elucidate the problem by starving the subject after the superimposition of some definite protein and following the nitrogen and sulphur outputs.

The experiments described in this paper fall into four groups. These were all carried out on myself, a healthy subject weighing 60 kg. The first series was designed to find the effect of two different proteins on the nitrogen output during the first few days of starvation. In order to eliminate the effect of previous food a nitrogen-free diet was taken until the nitrogen excretion for 24 hours was below 3 g., which is about the lowest that can be reached. Then the protein to be investigated was superimposed for one day. Two days of starvation followed and on the third day the basal diet was resumed until the nitrogen output had fallen to the original value. In order to have a basis of comparison a starvation experiment was carried out without the superimposition of protein: this constituted Exp. 1 (Table I). In Exps. 2 and 3 gelatin and egg white respectively were the proteins superimposed. These two proteins were chosen because of the marked contrast in their sulphur content or S : N ratio. The nitrogen-free diet consisted of olive oil, 100 g., tapioca, 300 g., sugar, 100 g.

The second group consisted of two experiments in which gelatin and egg white were superimposed on a basal diet containing 6 g. nitrogen. The third group was the same except that the basal diet contained about 11 g. nitrogen. The fourth was the same as the first except that no starvation followed the taking of the proteins. In all these experiments the 24-hour output of urine was collected under toluene and analysed on the following day. Total nitrogen was determined by the Kjeldahl method, urea and ammonia by the urease method, phosphorus by the uranium acetate method and uric acid by the Hopkins-Folin method.

Referring to Table I it will be noted that the urinary constituents fell more or less evenly for the first few days. Taking the S : N ratios, however, a distinct rise is seen from 1 : 15·8 on the first day to 1 : 12·3 on the day before starvation. This means that when the body is on a nitrogen-free diet and

Table I.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
9 Jan.	Basal	4·788	0·3640	450	1·7892	0·3022	1 : 15·8	1 : 2·6
10 ,,	,, ...	4·382	0·4134	327	1·8316	0·2748	1 : 15·9	1 : 2·3
11 ,,	,, ...	3·052	0·3080	212	1·4200	0·2528	1 : 12·0	1 : 2·1
12 ,,	,, ...	2·996	0·3024	210	1·1644	0·2418	1 : 12·3	1 : 2·5
13 ,,	Starving (Exp. 1)	2·492	0·1848	205	0·7524	0·2143	1 : 11·6	1 : 3·3
14 ,,	,, (,,)	4·872	0·3360	290	1·3488	0·4286	1 : 11·3	1 : 3·6
15 ,,	Basal	6·944	0·3808	337	1·2498	0·4012	1 : 17·3	1 : 5·5
16 ,,	,, ...	4·480	0·3966	225	1·4340	0·2638	1 : 16·9	1 : 3·1
17 ,,	,, ...	2·996	0·3920	257	1·1369	0·2857	1 : 10·4	1 : 2·6
18 ,,	Gel. supsd (Exp. 2)	5·901	0·5460	307	0·8520	0·2473	1 : 23·8	1 : 6·9
19 ,,	Starving	3·984	0·2352	327	1·0648	0·2693	1 : 11·0	1 : 3·7
20 ,,	,, ...	6·804	0·3024	302	1·4624	0·4836	1 : 14·0	1 : 4·6
21 ,,	Basal	8·284	0·5656	437	1·0224	0·5660	1 : 14·5	1 : 8·1
22 ,,	,, ...	4·228	0·3024	262	0·9088	0·2528	1 : 16·7	1 : 4·6
23 ,,	,, ...	3·332	0·2912	285	0·9228	0·2253	1 : 14·7	1 : 3·6
24 ,,	Alb. supsd (Exp. 3)	3·920	0·4088	312	0·7952	0·4876	1 : 8·0	1 : 4·9
25 ,,	Starving	5·628	0·2800	365	0·7808	0·5660	1 : 9·9	1 : 7·3
26 ,,	,, ...	7·112	0·3360	292	1·4484	0·4506	1 : 15·7	1 : 4·9
27 ,,	Basal	7·532	0·4368	415	1·1070	0·5056	1 : 14·8	1 : 6·8
28 ,,	,, ...	3·900	0·2912	299	0·8662	0·2693	1 : 16·7	1 : 4·5
29 ,,	,, ...	2·996	0·2856	270	0·9230	0·2583	1 : 11·5	1 : 3·2

drawing on its tissue protein there is either a breakdown of some sulphur-rich protein or the retention of the nitrogen of some protein of average sulphur content, *e.g.* muscle. If, however, there were a breakdown of, say, muscle tissue along with a nitrogen retention, this nitrogen should ultimately be excreted and bring down the S : N ratio. This is exactly what happened, as, on the return to the basal diet, the nitrogen rose almost 50 %, while the sulphur output fell a little. Corresponding to this the S : N ratio fell to 1 : 17, but by Jan. 17th rose again to 1 : 10·4. If the total nitrogen and sulphur output of the two starvation and two following days be taken, the S : N ratio of the sum is 1 : 14·3, which corresponds to the average value found for muscle. Apparently then there is either an alternating breakdown of sulphur-rich and sulphur-poor protein or else a breakdown of some protein such as muscle with a nitrogen retention. The sudden elimination of the retained nitrogen on the first day after starvation is probably due to the stimulating action of the food.

Had food been taken constantly the excretion of this retained nitrogen would have increased until the S : N ratio was 1 : 14·3. At this point the body would have a certain quantity of temporarily retained nitrogen in it but its excretion would equal the rate of formation and hence a S : N ratio of 1 : 14·3 would result if muscle were the source. The same rise in the S : N ratio was seen in Cathcart's subject [1907]. The S : N ratio rose from 1 : 14·7 on the last day of starvation to 1 : 9·9 on the third day of a nitrogen-free diet.

Both these experiments, however, show that the influence of carbohydrate on the sulphur moiety is more marked than on that of the nitrogen. This is to be expected, for, if the sulphur of the protein molecule is the first to be broken down and eliminated, the sparing effect of carbohydrate on protein breakdown should be directed primarily at the sulphur-containing group.

From the data of Exp. 1 it is possible to calculate the nitrogen and sulphur loss due to starvation alone by subtracting the basal value (the nitrogen and sulphur output on the day before starvation) from the total nitrogen for each day on which the nitrogen output exceeds the basal value. In this case the two starvation and two subsequent days are involved. The first starvation day gives a positive balance however.

Excess nitrogen output above basal value = 6·804 g.
,, sulphur ,, ,, ,, ,, = 0·3646 g.
S : N = 1 : 18·6.

This low ratio does not necessarily mean that sulphur-poor protein has been used for starvation metabolism. The explanation is that on the one hand the S : N ratio of the basal value is high, which shows up the early excretion of sulphur and the retention of the nitrogen. The S : N ratio of 1 : 18·6 on the other hand shows up the elimination of the retained nitrogen. As was stated above, the S : N ratio of those four days is 1 : 14·3. The conclusions drawn from this experiment are:

1. That the sulphur fraction of the protein molecule is broken down and eliminated in advance of the nitrogen. This applies to the breakdown of tissue protein when on a carbohydrate-fat diet.

2. The sparing action of carbohydrates on the breakdown of body protein is exerted first on the sulphur-containing moiety. Carbohydrate has no effect on the temporarily retained nitrogen and this cannot be used for synthesis as there is no adequate supply of sulphur present to make up the full complement necessary for the formation of protein.

The same procedure was carried out in Exp. 2, with the exception that 70 g. gelatin containing 11·8 g. nitrogen and 0·3367 g. sulphur with a S : N ratio of 1 : 35 were superimposed on the day before starvation. It will be noted that the same basal value as in Exp. 1 had been reached on the day before gelatin was taken. Gelatin was chosen as a type of sulphur-poor protein, as will be seen from the S : N ratio. On the day of superimposition the S : N ratio fell to 1 : 23 instead of rising. There is no advanced output of sulphur even, although the S : N ratio for the day (1 : 23) is higher than that of the gelatin taken, because the total sulphur output on the day of superimposition is actually lower than that of the previous day. The sulphur has been completely stored, while some of the nitrogen has been eliminated. On the first starvation day the S : N ratio showed a sharp rise to 1 : 11. The probable explanation of this change is that the first day of starvation produces a very definite reduction in the material metabolised. The nitrogen drops considerably, while the sulphur shows a slight rise. This slight rise is probably due to the metabolism of protein stored the previous day—possibly in an unstable form. Evidence of such an instability will be seen in later experiments. It is not considered, however, that the low S : N ratio on the day of superimposition and the high ratio the following day are to be attributed to a sulphur lag.

The explanation suggested is that in the storage of protein the sulphur fraction is stored first and the nitrogen moiety later. In other words it is the theory of the nitrogen lag applied to the anabolism of protein as well as to its catabolism. One can imagine the movements of the nitrogen fraction always following those of the sulphur. In protein catabolism the sulphur is eliminated first and then the nitrogen follows, whereas in protein anabolism the sulphur is built up first, then the nitrogen. When the influence of carbohydrates on the breakdown of body protein is considered, the effect is seen first on the sulphur output and later on the nitrogen output (Exp. 1). It will be noted that on the return to the basal diet the nitrogen and sulphur outputs both rose proportionately, as there was little change in the S:N ratio. The S:N ratio fell, however, the next day to 1:16·7, so that evidently there was some retained labile nitrogen in the body, though the S : N ratio never returned to its original high value. It is probable that the intake of sulphur-poor protein in the form of gelatin has saturated the tissues to their maximum capacity for holding sulphur-free nitrogen bodies; hence the failure to reach the previous high value of the S : N ratio. If these figures are treated as in Exp. 1 we find:

Excess nitrogen above basal value = 14·557 g.
,, ,, ,, ,, ,, in Exp. 1 (pure starvation) = 6·804 ,,
,, ,, ,, that in Exp. 1 = 14·557 – 6·804 = 7·753 ,,

Hence by taking 11·8 g. nitrogen in the form of gelatin before starving, 7·753 g. nitrogen were excreted above the output due to starvation alone, and hence 4·047 g. nitrogen were stored or utilised to cover the breakdown of body protein in starvation. In the case of the sulphur output the same calculation is made.

Excess sulphur above basal value = 0·3704 g.
,, ,, ,, ,, for Exp. 1 = 0·3646 ,,
,, ,, ,, that in Exp. 1 = 0·0580 ,,

As 0·3363 g. were ingested it follows that 0·2783 g. were stored, or covered the sulphur output in starvation. The S : N ratio of the stored material is 1 : 14·5, while that of the gelatin was 1 : 35. If one be permitted to draw a deduction from the nitrogen and sulphur content there would seem to have been a selection among the various amino acids in gelatin in order to retain a substance corresponding to muscle protein.

In the case of phosphorus the total output in Exp. 2 is less by 0·0288 g. P_2O_5 than in Exp. 1. Taking the total nitrogen output in Exp. 1 from the day before starvation to the second post day we find it to amount to 21·77 g. nitrogen. In Exp. 2 it amounts to 29·8 g. nitrogen, but as 11·8 g. nitrogen were ingested the real loss to the body is 29·8 – 11·8 = 18 g. This is 3·77 g. nitrogen less than in Exp. 1; the stored phosphorus is 0·0288 g. P_2O_5 and the P_2O_5 : N ratio of this is 1 : 130. From these figures it can be seen that 58 % of the excess nitrogen output due to starvation can be spared by gelatin.

This is a higher value than those found by the older workers, and is possibly due to the different method of calculation employed. The method of calculation used in the present experiments narrows the question to finding the effect of gelatin on the excess output of nitrogen above the basal value due to starvation alone.

Experiments on the capacity of gelatin to cover the minimum wear and tear output on a nitrogen-free diet were carried out by Robison [1922]. He found that gelatin had very little sparing effect even although a large excess was taken. It would seem to follow that protein of a more specific nature is required to cover the endogenous wear and tear than to cover the excess output above this occasioned by starvation.

Exp. 3 consisted in superimposing egg white under the same conditions. Unfortunately in this experiment the basal value was a little higher on the day before superimposition than on the corresponding day of the previous experiment. 495 g. egg white, containing 8·87 g. nitrogen and 0·960 g. sulphur, with a S : N ratio of 1 : 9·2, were distributed evenly over the three meals on the day of superimposition. The egg white was obtained directly from fresh eggs and taken in the soft-boiled state. It will be noted that this is a sulphur-rich protein. On the day of superimposition the nitrogen output rose 0·59 g., while the sulphur output was nearly double that of the previous day. The S : N ratio accordingly rose to 1 : 8. On the first day of starvation the sulphur and nitrogen outputs both rose, which is in marked contrast to Exps. 1 and 2, where a fall was the rule. A possible explanation of this variation will be found in some of the later experiments. It is probably associated with a peculiar instability of egg white. The S : N ratio on this day is still high. On the second day of starvation the S : N ratio fell to 1 : 15·7, the sulphur output falling a little, while the nitrogen output rose. On the first post day, when the basal diet was resumed, there was the same rise in the nitrogen output though much less than in the previous experiments. The sulphur output also rose a little, but the S : N ratio fell to 16·7, apparently an excess output of nitrogen in relation to sulphur. On the last day the ratio rose to 11·5. In all three experiments a certain amount of temporarily retained nitrogen seems to be eliminated after starvation, but in Exps. 2 and 3 this occurs on the second post day, as deduced from the low S : N ratio, whilst in Exp. 1 it occurs mostly on the first post day. Owing to the fact that the original basal value was not reached in this experiment, there are two figures on which to base the calculations, either the actual basal value obtained for this experiment or the original one of 2·9 g. nitrogen. Taking the former we get:

Excess nitrogen above basal (3·3 g.) = 11·432 g.
,, ,, ,, ,, in Exp. 1 = 6·804 ,,
,, ,, ,, that in Exp. 1 = 11·432 − 6·804 = 4·628 ,,

Hence 8·87 g. nitrogen in the form of egg white caused an excess output above that found in pure starvation of 4·628 g. nitrogen. As 8·87 g. nitrogen were ingested it follows that 4·142 g. nitrogen were stored, or covered the breakdown of body protein during starvation.

Excess sulphur above basal = 1·1792 g.
,, ,, ,, ,, in Exp. 1 = 0·3646 ,,
,, ,, ,, output in Exp. 1 = 0·8146 ,,

As 0·960 g. sulphur was ingested, 0·1454 g. was retained or covered the breakdown of body sulphur. The S : N ratio of the retained material is 1 : 31·7 —a low value. The storage of sulphur is only 0·1454 g. in this experiment, while in Exp. 2 with gelatin it was almost twice as much. It is rather an anomaly that the sulphur retention should have been so low with the sulphur-rich protein as compared with the sulphur-poor gelatin. It is unlikely that some other limiting factor is associated with egg white, such as a minimal percentage of some essential amino acid, especially as it is a much more perfect protein than gelatin. There is the possibility, however, that egg white brought about the temporary retention of some unstable sulphur-rich protein which was quickly broken down. It has recently been shown to contain only 15 % of its sulphur in the form of cystine, so that the effective content of egg white in this amino acid might be low [Harris, 1923]. This presupposes that cystine is the only form of sulphur which the body can utilise. It is questionable however if the sulphur in gelatin is in the form of cystine [Dakin, 1920], though it was, nevertheless, well utilised. The percentage retention of the nitrogen of the white was nearly 50 %, whilst in the case of gelatin it was only 31 %. There is, however, an inconsistency in the above calculations. The excess for this experiment was calculated with 3·3 g. nitrogen as a basal value. The excess output was then compared with the control Exp. 1 in order to see the effect of the egg white on starvation. The excess output on pure starvation (Exp. 1) was calculated on a basal figure of 2·9 g. nitrogen. Hence, to make the comparison more valid, the excess output for pure starvation should be recalculated with 3·3 g. nitrogen as a basal figure. This result should then be subtracted from the excess output found in Exp. 3 with 3·3 g. nitrogen as a basal figure. This recalculation gave an excess output for pure starvation of 5·66 g. nitrogen. The excess output above this in Exp. 3 then becomes 3·098 g. nitrogen: applying the same method to the sulphur we find that 0·1867 g. was stored. The S : N ratio of this stored material is 1 : 16·5, which appears more reasonable than the ratio based on the first calculation. If we take 2·9 g. as the basal figure, a storage of 2·562 g. nitrogen and 0·2960 g. sulphur is found. The S : N ratio in this case is 1 : 9·6. This would bear out the hypothesis that the body stores sulphur first.

These three methods of calculation have each yielded a different S : N ratio, namely, 1 : 31·7, 1 : 16·5 and 1 : 9·6. It is most likely that the value 1 : 16·5 is the correct one but it is possible that the long period of nitrogen starvation may have so altered conditions that a sulphur-rich or a sulphur-poor protein has been stored. From the last two experiments it cannot be definitely stated whether the body stores protein whose composition corresponds to that of the food on the one hand or to that of muscle on the other. On the whole the evidence from Exp. 2 with gelatin favours the view that the protein stored corresponds in its sulphur content to muscle tissue. The same can be said of Exp. 3 if we accept the basis of calculation which gives a S : N ratio of 1 : 16·5. There is a certain amount of evidence that in the process of storing the sulphur may be retained first and the nitrogen later, and thus, although the end result is to have a protein of average S : N ratio, for a short time during storage a sulphur-rich protein may exist; and *vice versâ* in the breakdown of protein.

Table II.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
23 Feb.	Basal	5·460	0·3472	280	1·491	0·3737	1 : 14·6	1 : 3·6
24 ,,	,, ...	5·544	0·3640	322	1·526	0·3242	1 : 17·1	1 : 3·6
25 ,,	,, ...	5·768	0·4480	302	1·562	0·4396	1 : 11·9	1 : 3·6
26 ,,	,, ...	5·537	0·4256	370	1·278	0·4836	1 : 11·1	1 : 4·0
27 ,,	,, ...	6·552	0·3752	467	1·278	0·5056	1 : 12·9	1 : 5·1
28 ,,	Gel. supsd (Exp. 4)	9·240	0·6992	457	1·313	0·7969	1 : 11·5	1 : 7·03
29 ,,	Basal	6·860	0·5992	312	1·171	0·5605	1 : 12·2	1 : 4·0
1 March	,,	6·468	0·6608	322	1·242	0·4461	1 : 14·4	1 : 5·2
2 ,,	,,	6·244	0·6104	462	1·207	0·4561	1 : 13·6	1 : 5·1
3 ,,	,,	6·076	0·5040	407	1·936	0·4561	1 : 13·6	1 : 5·8
4 ,,	Alb. supsd (Exp. 5)	7·252	0·4984	462	1·312	0·7912	1 : 9·1	1 : 5·5
5 ,,	Basal (,,)	8·344	0·5432	565	1·312	0·7473	1 : 11·1	1 : 6·3
6 ,,	,,	6·076	0·4816	360	1·278	0·4467	1 : 13·6	1 : 4·7
7 ,,	,,	6·272	0·4312	542	1·3845	0·4506	1 : 13·9	1 : 4·5

Exps. 4 and 5 were carried out with the same two proteins, superimposed in this case on a diet containing about 6·4 g. nitrogen, but without subsequent starvation. In Exp. 4, where the nitrogen output on the pre-days is unfortunately rather irregular, 70 g. gelatin containing 9·939 g. nitrogen and 0·3750 g. sulphur with a S : N ratio of 1 : 26·5 were superimposed. The S : N ratio was 1 : 12·9 on the day before superimposition (Table II). This ratio rose on the day gelatin was taken, which is in contrast with Exp. 2. The ratio rose to 1 : 11·5. The nitrogen and sulphur outputs both fell to their basal value by the fourth day, whilst the S : N ratio fell a little below its basal value. It will be noted that the S : N ratio was highest on the day of superimposition and lowest on the third day, so that there was clearly a nitrogen lag. The output of both nitrogen and sulphur was highest on the day of superimposition. The balance is calculated by subtracting the total nitrogen output on this diet from the output on the day of superimposition and subsequent days.

Nitrogen excreted in excess of basal = 2·996 g.
,, ingested = 9·939 ,,
,, retained = 9·939 – 2·996 = 6·943 ,,
Sulphur excreted in excess of basal = 0·3459 ,,
,, ingested = 0·3750 ,,
Sulphur retained = 0·3750 – 0·3459 = 0·0291 ,,
S : N ratio of stored material = 1 : 22·8.

In this case the material stored from gelatin is much poorer in sulphur than in Exp. 2. The conditions however are not quite identical. In Exp. 2 the body had lost protein and was in a relatively sulphur-poor condition, as is seen from the high S : N ratio before the gelatin was superimposed. In Exp. 4, on the other hand, the body is in nitrogen equilibrium with a protein of high sulphur content as is seen from the high S : N ratio on the day before superimposition (1 : 12·9). It is possible that the body was relatively saturated with sulphur groups and retained the nitrogen of the gelatin in order to lower the S : N ratio of the circulating protein to an average.

It will be noted that 6·9 g. of the 9·9 g. nitrogen of the gelatin ingested was retained. This is a high value for gelatin, which is considered to be a protein of low biological value, so that in all probability the basal diet improved the quality of the gelatin as a protein and made it suitable for storage.

Exp. 5 was carried out in the same way with egg white, 500 g. of this containing 19·296 g. nitrogen and 1·059 g. sulphur, with a S : N ratio of 1 : 9·6, being superimposed. On the day of superimposition the nitrogen output rose only 1·2 g., whilst the sulphur output did not exceed that on the day gelatin was taken; nevertheless the S : N ratio rose to 1 : 9·1. On the next day the nitrogen output rose 2·3 g. above the basal value, whilst the sulphur output and the S : N ratio fell a little. The maximum output of nitrogen is on the second day in contrast to what was seen in Exp. 4. This late rise seems to prove the extreme lability of the material stored from egg white. This also probably accounts for the rise in nitrogen output in Exp. 3 on the first day of starvation, instead of the fall seen in Exps. 1 and 2. This lability is possibly conditioned partly by the high sulphur content and partly perhaps by the nature of the sulphur moiety of egg white. The material stored and broken down again is evidently sulphur-rich, as the S : N ratio is high for the first two days, whilst on the third day, when the nitrogen output has reached its basal figure, the S : N ratio is 1 : 13·6, a little below the average for the experiment. It will also be noted that the sulphur has preceded the nitrogen output. The following figures show the balance.

Nitrogen excreted above the basal = 3·440 g.
,, ingested = 10·206 ,,
,, retained = 10·206 − 3·440 = 6·766 ,,
Sulphur excreted above the basal = 0·6262 ,,
,, ingested = 1·059 ,,
,, retained = 1·059 − 0·6262 = 0·4332 ,,
S : N ratio of the stored material = 1 : 15·2.

In this experiment there has been a much better utilisation of sulphur than in Exp. 2. Again the body has selectively retained a material corresponding in its nitrogen and sulphur content to muscle tissue. It is necessary to remark that, although 0·4332 g. sulphur was retained, it does not follow that it was the sulphur of the egg white. It is quite possible that most of the egg white sulphur was excreted and the sulphur of the basal diet stored in order to build up a protein with the nitrogen of the egg white. A later experiment lends support to this view.

Table III.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
22 March	Basal	11·116	0·504	495	1·948	0·610	1 : 18·2	1 : 5·7
23 ,,	,, ...	10·500	0·504	440	1·5713	0·560	1 : 18·7	1 : 6·6
24 ,,	,, ...	10·416	0·5152	482	1·929	0·599	1 : 17·3	1 : 5·3
25 ,,	,, ...	11·284	0·470	462	2·214	0·588	1 : 19·1	1 : 5·0
26 ,,	Gel. supsd (Exp. 6)	16·520	0·739	607	2·692	0·901	1 : 18·3	1 : 6·1
27 ,,	Basal	14·448	0·750	600	2·258	0·626	1 : 23·0	1 : 6·3
28 ,,	,,	13·272	0·694	565	2·498	0·670	1 : 19·7	1 : 5·3
29 ,,	,,	11·200	0·560	487	2·857	0·615	1 : 18·1	1 : 3·9
30 ,,	,,	11·680	0·539	467	2·333	0·582	1 : 20·0	1 : 5·0
31 ,,	Alb. supsd (Exp. 7)	13·888	0·649	590	2·491	1·198	1 : 11·6	1 : 5·5
1 April	Basal	15·232	0·772	565	2·737	1·187	1 : 12·8	1 : 5·7
2 ,,	,,	11·312	0·694	402	1·944	0·7364	1 : 15·3	1 : 5·7
3 ,,	,,	11·704	0·660	467	1·884	0·747	1 : 15·6	1 : 6·7

Exps. 6 and 7 (Table III) were the same as the preceding, but the basal diet was much richer in nitrogen. In Exp. 6 gelatin was taken in the same quantity as in Exp. 4. The average S : N ratio of the pre-days was 1 : 18·3, but the actual value on the day before superimposition was 1 : 19·1. On the day of superimposition it rose to its average value of 1 : 18·3. On the next day it fell to 1 : 23 and had returned to its average value two days later. It will be noted that there was a distinct nitrogen lag. The maximum output of nitrogen occurred on the day of superimposition and four days elapsed before it fell to the basal value. Five days elapsed before the sulphur came down to its basal value. The following is the balance for the period.

Nitrogen excreted above the basal value = 10·574 g.
,, ingested = 9·939 ,,
,, lost = 10·574 − 9·939 = 0·635 ,,
Sulphur excreted above the basal value = 0·4605 ,,
,, ingested = 0·3750 ,,
,, lost = 0·4605 − 0·3750 = 0·0850 ,,
S : N ratio of material lost = 1 : 10·2.

This small loss of both nitrogen and sulphur may either be real and due to the stimulating effect of gelatin on protein metabolism such as was observed by Cathcart and Green [1913], or it may simply fall within the limits of experimental error. The most important point is that when gelatin is superimposed on a basal diet of high nitrogen content, it is apparently completely rejected. In Exp. 7, 500 g. egg white containing 9·8 g. nitrogen and 1·204 g. sulphur with a S : N ratio of 1 : 11·6 were superimposed. On the day of superimposition the output of sulphur was nearly doubled, whilst that of the nitrogen rose only 2·4 g. The S : N ratio correspondingly rose to 1 : 11·6, almost double that of the pre-day (1 : 20). On the next day there is a repetition of what was seen in Exp. 5, the sulphur output falling a little whilst the nitrogen output rose nearly 4 g. The same sharp fall is seen on the subsequent day but the sulphur never returned to its basal value. The S : N ratio continued to fall but failed to reach the original value. Taking as the basal value an average of the pre-days as in Exp. 7, there is the following balance:

Nitrogen excreted above the basal value = 7·0 g.
,, ingested = 9·8 ,,
,, stored = 9·8 – 7·0 = 2·8 ,,
Sulphur excreted above the basal value = 1·5171 g.
,, ingested = 1·2040 ,,
,, lost = 1·5171 – 1·2040 = 0·3131 ,,

In this case there is a considerable loss in sulphur with a small retention of nitrogen. It will be noted that the best retention of egg white both as regards the nitrogen and the sulphur was in Exp. 5, where the basal diet contained about 6 g. nitrogen. Possibly at the high level of protein intake in this experiment the body is at its maximum capacity for retaining nitrogen. In Exps. 4 and 5 the total nitrogen of the faeces rose in each case (about 1 g. above the average) on the day after superimposition. In Exps. 6 and 7, where the daily average output in the faeces was 2·236 g., the rise was rather more than 1 g. This increase in the nitrogen of the faeces may be due to non-absorption, in which case there would probably have been a lack of absorption of a corresponding quantity of sulphur. This would reduce the calculated quantity of material stored, but its quality would remain as found.

Table IV.

Date	Diet	Total N g.	Ammonia N g.	Uric acid mg.	Total P (as P_2O_5) g.	Total S g.	S : N	P_2O_5 : N
14 April	Basal	4·312	0·408	327	2·101	0·3902	1 : 10·9	1 : 2·0
15 ,,	,,	4·256	0·3584	412	1·505	0·2528	1 : 16·8	1 : 2·8
16 ,,	,,	3·276	0·358	360	1·177	0·1978	1 : 16·5	1 : 2·7
17 ,,	,,	3·024	0·364	350	1·079	0·2088	1 : 14·4	1 : 2·8
18 ,,	,,	2·688	0·336	336	1·079	0·2088	1 : 12·8	1 : 2·4
19 ,,	Gel. supsd (Exp. 8)	4·564	0·476	345	0·568	0·4506	1 : 10·1	1 : 8·0
20 ,,	Basal	4·340	0·442	370	0·837	0·2528	1 : 17·1	1 : 5·1
21 ,,	,,	3·668	0·341	445	0·894	0·2528	1 : 14·4	1 : 4·1
22 ,,	,,	3·192	0·285	370	0·695	0·2308	1 : 13·8	1 : 4·5
23 ,,	,,	2·912	0·280	345	0·624	0·2253	1 : 12·9	1 : 4·6
24 ,,	Alb. supsd (Exp. 9)	3·696	0·330	472	0·781	0·4506	1 : 8·2	1 : 4·7
25 ,,	Basal	3·528	0·375	370	0·454	0·5001	1 : 7·0	1 : 7·7
26 ,,	,,	3·836	0·383	332	0·695	0·3792	1 : 10·1	1 : 5·5
27 ,,	,,	3·388	0·296	420	0·569	0·3627	1 : 9·3	1 : 5·6
28 ,,	,,	3·276	0·263	375	0·582	0·3462	1 : 9·4	1 : 5·6

The last group of experiments (Exps. 8 and 9, Table IV) were carried out with the nitrogen-free basal diet employed in the first group of experiments. The experiments were exactly similar to the earlier ones, except that there was no subsequent starvation. In Exp. 8, 70 g. gelatin, containing 9·939 g. nitrogen and 0·3705 g. sulphur, with a S : N ratio of 1 : 26·5, were superimposed when the nitrogen output was below 3 g. It will be noted that there was the same tendency for the S : N ratio to rise on the nitrogen-free diet.

On the day of superimposition the ratio rose to 1 : 10·1, the reverse of what was noted in Exp. 2. The possible reason of this is that in Exp. 2 the body had been on a nitrogen-free diet for seven days and, in addition, had starved two days. Under such conditions the tissues would store protein more readily. The following balance was found:

Nitrogen excreted above the basal value = 5·236 g.
,, ingested = 9·939 ,,
,, stored = 9·939 – 5·236 = 4·703 ,,
Sulphur excreted above the basal value = 0·3683 ,,
,, ingested = 0·3705 ,,
,, stored = 0·3705 – 0·3683 = 0·0022 ,,

In this case the nitrogen only has been stored. It is interesting to compare this experiment with Exp. 4, where the same quantity of gelatin was superimposed on a basal diet containing 6 g. nitrogen. In Exp. 4, 6·643 g. nitrogen and 0·0291 g. sulphur were stored. *A priori* the body should have retained more of the gelatin on a nitrogen-free diet than on a nitrogen-containing one. The reason for the poorer retention possibly lies in the fact that the basal diet in Exp. 4 may have improved the quality of the gelatin. It is also to be noted that even on the fifth day after superimposition the nitrogen had not quite returned to its basal value. This seems to show that the tissues were in need of nitrogen and were trying to utilise to the utmost an imperfect protein. The early excretion of sulphur with nitrogen lag is again seen in this experiment. In Exp. 9 egg white, containing 9·9619 g. nitrogen and 0·9995 g. sulphur with a S : N ratio of 1 : 9·9, were superimposed. Taking 2·912 g. nitrogen as the basal output we get:

Nitrogen excreted above the basal value = 1·0218 g.
,, ingested = 9·9619 ,,
,, stored = 9·9619 – 1·0218 = 8·9401 ,,
Sulphur excreted above the basal value = 0·9123 ,,
,, ingested = 0·9995 ,,
,, stored = 0·9995 – 0·9123 = 0·0872 ,,
S : N ratio of stored material = 1 : 101.

Again there is a poor retention of sulphur. The sulphur retention to cover starvation output is much greater than this in Exp. 3. The best retention of the sulphur of egg white was in Exp. 5, where 0·4322 g. was stored. In Exp. 5, however, it is likely that it was really the sulphur of the basal diet that was retained in order to build up some protein with the nitrogen of the egg white. In such a case the sulphur of the white may have been almost completely eliminated. The figures in Exp. 5 show a retention of 0·4322 g. sulphur when the white was superimposed, but clearly it does not necessarily follow that it was actually the sulphur of the white which was retained.

In fact from Exp. 9 it would seem as if the real value of the sulphur of egg white was low. Exp. 9 is a stringent experiment as the tissues were in great need of protein as compared with Exp. 5. In Exp. 9 the retention of nitrogen, however, was nearly 90 % of the intake.

This nitrogen was held firmly as the nitrogen output had not reached its basal value by the fifth day after superimposition, when the experiment was stopped as the diet became rather trying to take. The extreme difference in the quality of the material stored will be noted in Exps. 5 and 9. In Exp. 5 the material stored had a S : N ratio of 1 : 15·2, corresponding to the average for muscle tissue, whilst in Exp. 9 the retained material consisted of nitrogen alone (S : N = 1 : 101). It will be noted that the highest output of sulphur is on the day after superimposition, whilst in the case of the nitrogen it is on the second day after taking the egg white. The same lability of the material stored is seen as in Exps. 5 and 7, but, owing to the poverty in nitrogen of the body the material is retained a day longer than in the two previous experiments. This experiment also shows clearly the delay in the nitrogen excretion after that of sulphur. The sulphur output never reached its basal value although it had been eliminated almost completely.

The results of these last two experiments taken in conjunction with Exps. 4 and 5 show the effect of a basal diet in improving the quality of such proteins as gelatin and egg white in such a way as to render them more suitable for storage.

Throughout all these experiments the output of uric acid was followed. In general it will be noted that the lowest output of uric acid was on the nitrogen-free diet, and the highest on the high-nitrogen diet. The total variation in the uric acid output is nearly 300 %, the lowest output being 205 mg. on the first day of starvation, and the highest, 600 mg. on the day gelatin was taken in Exp. 6. The output in general varies with the level of nitrogen equilibrium at the time. This indicates that one of the factors governing the output of uric acid must be the metabolism not only of the ingested protein but also of the ingested carbohydrate and fat. On returning to the nitrogen-free diet after starving a rise in uric acid output is seen. According to Cathcart the presence of carbohydrate is supposed to aid in the synthesis of uric acid or its precursor from some nitrogenous source, possibly arginine and histidine [Ackroyd and Hopkins, 1916]. Folin classed uric acid as the product of a more or less constant metabolic process which was little influenced by food ingestion. This position can possibly be held for creatinine which appears to be constant in output, whether on a high- or low-nitrogen diet. The function associated with creatinine formation is probably one which must go on at all costs and within narrow limits. The output of uric acid, however, appears to be intimately associated with the metabolism of the three energy-yielding foodstuffs.

Conclusions.

The first question to be answered was whether there was a delay in the excretion of ingested protein, such as might lead to the conclusion that it was stored. This delay has been observed in the case both of gelatin and egg white. The ease with which these materials are stored and the duration of their retention in the body varies, however, with the nutritive condition at the time. In Exp. 4, 60 % of the gelatin was stored and the remaining 40 % was all eliminated in three days. This was with nitrogen equilibrium in the neighbourhood of 6 g. *per diem*. In Exp. 6, with nitrogen equilibrium at 11 g. *per diem*, the whole of the gelatin was eliminated in three days. In Exp. 8, where the body was on a nitrogen-free diet and when it was in the poorest nutritive condition as regards nitrogen of all three experiments, the amount stored was less than 50 %. It will be noted that it took at least five days for the rejected part to be eliminated. The possible explanation of this variation may be that in Exp. 6 the body was flushed to its maximum capacity with protein and was incapable of storing any more. In Exp. 4 the nitrogen condition of the body was below its optimum so that gelatin was stored and in addition its quality was improved by the nitrogen of the basal diet. In Exp. 8 the tissues, being poor in nitrogen, retained as much of it as possible. The amount stored was less than in Exp. 4 with a nitrogen-containing diet, because there was no protein in the basal diet to improve its quality for storage.

As regards the egg white it was best retained in Exp. 9, where the body was in need of protein, 90 % of the nitrogen intake being retained. The storage of sulphur, however, is different. In Exp. 9 the S : N ratio of the stored material is 1 : 101, practically no sulphur being retained. In Exp. 5 the S : N ratio of the stored material is 1 : 15·1, 40 % of the sulphur being retained. It was probably the sulphur of the basal diet which was mostly retained. The main points to be noted are:

1. The nitrogen of egg white can be stored independently of the sulphur, hence it follows that a sulphur-poor material may be retained in the tissues.
2. If the protein superimposed contain sulphur of proper quality much sulphur may be retained along with the nitrogen ingested to form a material whose S : N ratio is similar to that of tissue (muscle) protein.

It would seem to be clear from these experiments that the changes which occur in the protein molecule affect first the sulphur fraction and then the nitrogen. In the process of storage the sulphur is retained first and the nitrogen later. In breakdown the reverse occurs. If an attempt is made to visualise the possible changes which occur one cannot consider the sulphur-containing group as a nucleus around which sulphur-free nitrogen-containing bodies are built because in the catabolic phase one would require to assume that the sulphur moiety of the protein molecule would be attacked last and all the evidence is against such a view. Rather must the molecule be looked on as a chain of amino acids with the sulphur-containing group interposed at certain links and conferring lability on the molecule.

It has been argued that the early excretion of sulphur is due to the fact that the breaking off of the sulphur molecule precedes the deamination of the amino group. Wolf and Osterberg [1911], however, found that when cystine was administered to an animal the nitrogen was eliminated before the sulphur. The natural inference from this experiment is that the sulphur was protected until the amino group had been removed. Another fact, which appears to support the view that there is no causal relationship between the lag in the output of nitrogen as compared with sulphur and the process of deamination and oxidation, is that a similar discrepancy in output occurs when anabolic phenomena predominate, as in the storage or retention of protein within the body. In this storage phenomenon it must be noted, however, that the primary preference in retention is given to the sulphur part of the molecule. In view of the fact that this lag in the nitrogen is associated both with the anabolism of food protein and the catabolism of protein (tissue protein or food protein built up into some complex), but not with the catabolism of food protein in the amino acid form, it would seem to follow that, when a differential delay in excretion is observed after the ingestion of food protein, it may be assumed that this food protein must have been built up into some form of protein in the tissues and not immediately metabolised as amino acids.

In this connection it must be born in mind that the nitrogen and sulphur ingested in the form of protein are not excreted forthwith, but may be retained in the body for three to five days. Hence it must follow that the retention takes place in some protected form and the normal assumption is that it has been taken into the cells and retained there either to become an integral part of the cell content or simply to be added on to form a kind of prosthetic group. The main point is that it cannot be regarded as remaining and circulating in the tissue fluids in a highly labile form.

As regards the sparing effect of gelatin on the nitrogen output in starvation it was found that 58 % of the excess output due to starvation could be spared by gelatin. This result is much superior to those previously given and may be due simply to the methods of calculation adopted in the present series, although it is believed that the present value is nearer the truth than the older ones.

SUMMARY.

1. There is a delay in the excretion of ingested protein extending over several days. This occurs whether the basal diet contains nitrogen or not and even when the feeding is followed by a short period of starvation.

2. The sulphur moiety of the protein molecule is the first to be mobilised both in the storage and breakdown of protein. There is always a delay in the excretion of nitrogen as compared with sulphur. The delay in the excretion of sulphur, which is sometimes found, is not due to a delay in the utilisation of

the sulphur moiety of the protein. It is due to an active preferential retention of sulphur in the tissues.

3. There is a certain amount of evidence that the retained material can exercise a sparing action on protein breakdown in the first few days of starvation.

4. Ingested protein may be retained in the body in some relatively complex form.

I desire to acknowledge my deep indebtedness to Prof. E. P. Cathcart for much helpful advice and criticism during the progress of this work, which was done during the tenure of a Carnegie Scholarship.

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L. A NOTE ON THE KINETICS OF ENZYME ACTION.

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THE equation of Michaelis and Menten [1913] has been applied with success by Kuhn [1924] and others to numerous cases of enzyme action. It is therefore desirable to examine its theoretical basis. Consider the irreversible reaction $A \rightarrow B$, unimolecular as regards A, and catalysed by an enzyme. Suppose one molecule of A to combine reversibly with one of enzyme, the compound then changing irreversibly into free enzyme and B, where B may represent several molecules. We may represent this as:

$$\underset{(a-x)\,(e-p)}{A+E} \rightleftharpoons \underset{p}{AE} \rightarrow \underset{x}{B} + E.$$

Now let a be the initial concentration of A, e the total concentration of enzyme, x the concentration of B produced after time t, and p the concentration of enzyme combined with substrate at time t. We suppose e and p to be negligibly small compared with a and x. Then by the laws of mass action

$$\frac{dp}{dt} = k_1 (a - x)(e - p) - k_2 p - k_3 p,$$

where k_1, k_2, k_3 are the velocity constants of the reactions

$$A + E \rightarrow AE, \quad AE \rightarrow A + E, \quad \text{and} \quad AE \rightarrow B + E,$$

respectively. Now since p is always negligible compared with x and $a - x$, its rate of change must, except during the first instant of the reaction, be negligible compared with theirs.

For during the remainder of the reaction p diminishes from a value not exceeding e to zero, whilst x increases from zero to a. Thus the average value of $-\frac{dp}{dt} \div \frac{dx}{dt}$ is less than $\frac{e}{a}$. And provided $\frac{e}{a}$ is small it is clear that if the amount of combined enzyme decreased for a measurable time at a rate comparable with that of its substrate the reaction would come to an end. To take a concrete example Kuhn [1924] calculates that a yeast saccharase molecule at 15° and p_{H} 4·6 can invert 100 or more molecules of sucrose per second. Even if the enzyme concentration is so unusually large that the inversion of a strong sucrose solution is half completed in 10 minutes, $\frac{a}{e}$ cannot be less than 120,000, and if $-\frac{dp}{dt}$ attained 1 % of the value of $\frac{dx}{dt}$ for 1 second the reaction would stop owing to all the enzyme being set free. (Actually it may be shown that

$$\frac{dp}{dt}=\frac{-k_3(k_2+k_3)\,e\,(a-x)}{k_1\left(a-x+\frac{k_2+k_3}{k_1}\right)^3}.)$$

Hence $$k_1(a-x)(e-p)-k_2p-k_3p=0.$$

$$\therefore\ p=\frac{k_1e\,(a-x)}{k_2+k_3+k_1(a-x)}$$

$$=\frac{e\,(a-x)}{a-x+\frac{k_2+k_3}{k_1}}$$

$$\therefore\ \frac{dx}{dt}=k_3p=\frac{k_3e\,(a-x)}{a-x+\frac{k_2+k_3}{k_1}}.$$

This is Michaelis and Menten's [1913] equation, $(k_2+k_3)/k_1$ representing their constant K_s. They assume that the reaction

$$A+E\rightleftharpoons AE$$

is always practically in equilibrium, and K_s its equilibrium constant, *i.e.* that k_3 is negligible in comparison with k_2. Van Slyke and Cullen [1914] on the other hand assumed that the first stage of the reaction was irreversible, *i.e.* $k_2=0$, and arrived at the same equation. It is clear, however, that data as to the course of a reaction can give no indication of the ratio of k_2 and k_3, though when the velocity of the observed reaction, and hence k_3, is very large, the upper limit to k_2 deducible from the kinetic theory may possibly prove to be of the same order of magnitude.

It may be remarked that with this modification of their theory, Michaelis and Menten's analysis of the effects of the products of the reaction, or other substances which combine with the enzyme, still holds good.

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LI. HAEMOGLOBIN IN RELATION TO OTHER METALLO-HAEMATOPORPHYRINS

By ROBERT HILL.

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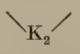
(*Received December 18th, 1924.*)

The object of this communication is to describe some observations which seem to throw some light on the union of the pigment to the protein in haemoglobin. The absorption spectra of different derivatives of haematoporphyrin are compared with those of haemoglobin, oxyhaemoglobin and haemochromogen. Then follow some experiments on the action of alkali on haemoglobin showing the formation of a pigment still containing the protein, but having the spectrum and properties of haemochromogen; on removal of the alkali the haemoglobin is regenerated. Finally some experiments on haemochromogen and on its ester are described showing their similarity in properties and the independence of the presence of water as a solvent in the formation of the compound of the ester with carbon monoxide.

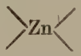
Many of the metallic derivatives of haematoporphyrin have been described and the close resemblance in spectrum between the zinc compound and oxyhaemoglobin has long been known [Schultz, 1904]. The tin compound was prepared by Milroy [1909] and is noteworthy for its great stability to acids, and for the sharpness of its absorption bands which far exceeds that of the other metallic compounds. In the present communication an aluminium compound is described which closely resembles the tin compound. The fact that the cobalt compound of haematoporphyrin having a two-banded spectrum can be reduced to a one-banded form was discovered by Laidlaw [1904]; it was found that the reduced cobalt compound was similar to the nickel compound and under the conditions here described actually showed two bands. The variety of properties and spectra shown by metallic derivatives of haematoporphyrin seems to deserve a collected description and a plate of spectra has been included.

It is apparent that three groups typified by acid haematoporphyrin, oxyhaemoglobin and haemochromogen, are present. They are independent of the valency of the metal in combination, as can be seen by noting the difference between silver and sodium, and the similarity between zinc, aluminium and tin. This could be explained most simply by supposing the spectrum to be an indication of the co-ordination number of the metal, with reference to the pigment. Starting with the above assumption an explanation of the differences in spectrum between haemochromogen, oxyhaemoglobin and haemoglobin, can be reached. Zinc and magnesium are given a co-ordination number of 4, nickel and copper 6. Hydrogen and the alkali metals are supposed to exhibit but a slight tendency to complex salt formation and form a group apart. It will be noted that the α band becomes less intense as we pass from sodium to potassium and haemoglobin itself can be regarded as the limit to this series. This would mean that in haemoglobin the iron is only attached to the pigment by its two normal valencies. When alkali acts on haemoglobin and the spectrum and properties of haemochromogen appear the iron is completely attached to the pigment and has a co-ordination number of 6. When the alkali is removed the spectrum and characteristic properties of haemoglobin are recovered; this seems to show a definite action of the globin on the iron atom, which is temporarily destroyed by the action of alkali. In oxyhaemoglobin, according to the spectrum, the iron would have a co-ordination number of 4 with reference to the pigment. If the oxygen molecule is combined with the iron atom, which has a co-ordination number of 6, no direct action of the globin on the iron atom could occur. This conclusion is supported by the fact that oxyhaemoglobin is not acted on by alkali under conditions which cause the formation of haemochromogen from haemoglobin. The fact that haemochromogen and its ester form carbon monoxide compounds leads to the supposition that there are two forms of haemochromogen, a form in which the iron is wholly combined with the pigment and which is oxidised to haematin, and a form similar to haemoglobin, which yields the carbon monoxide compound, and from which the iron is readily split off by acid. In "ordinary" haemochromogen, if there is an equilibrium between the two forms, it is easily seen that the action of oxygen must produce, in the end, nothing but haematin. In haemoglobin there can be under ordinary conditions no possibility of the oxidisible form of haemochromogen being produced. Haemochromogen, apart from globin, cannot, so far as is known, be made to show a haemoglobin spectrum; the action of globin is apparently specific. Conversion of the globin into metaprotein can readily take place without the pigment being split off; the compound (kathaemoglobin) on reduction has only the properties of haemochromogen as regards oxygen.

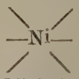
These assumptions can be represented graphically as follows:



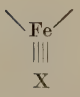
Potassium



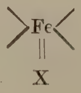
Zinc



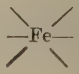
Nickel



Haemoglobin



Oxyhaemoglobin



Haemochromogen

1. METALLIC DERIVATIVES OF HAEMATOPORPHYRIN.

Preparation of haematoporphyrin. The haematoporphyrin used in the following experiments was prepared by the method of Nencki. After solution of recrystallised haemin in hydrobromic and acetic acids and hydrolysis of the addition product the pigment was precipitated from solution by adding concentrated sodium acetate solution containing about 10 % of tartrate. The pigment could then be readily filtered off and after washing with distilled water all the iron was found in the filtrate. The washed precipitate was dissolved in $N/20$ hydrochloric acid and reprecipitated with the minimum amount of Rochelle salt. This method of separating the iron was found preferable to the co-precipitation of the iron and pigment with sodium acetate, and separation from the gelatinous ferric hydroxide by solution in caustic soda.

Haematoporphyrin itself does not correspond with the iron-free pigment of haemoglobin; the relation of haematoporphyrin to the true iron-free pigment has been elucidated by H. Fischer and Müller [1925]. The difference in the position of the absorption bands between haemochromogen and the artificial compound from haematoporphyrin [Laidlaw, 1904] is very clearly shown in reference to the plate. The type of spectrum remains the same however; and when a qualitative study is being made haematoporphyrin serves for the purpose.

The introduction of metals. There are many methods of introducing a foreign metallic atom into the iron-free blood pigment, and different metals vary as to their ease of substitution. Of all the metals tried zinc was found to give the haematoporphyrin derivative most easily [Schultz, 1904]. The most general methods are: (1) to act on haematoporphyrin in glacial acetic acid with the acetate of the metal at 80°; (2) to heat the pigment to 80° for 5–10 minutes with an ammoniacal solution of a metallic salt. By method (1) manganese, iron (ferric), cobalt, nickel, copper, zinc and tin could be substituted; by method (2) iron (ferrous), cobalt, copper and lead. Magnesium can be introduced into haematoporphyrin ester by acting on it with the Grignard reagent, or the iron can be replaced directly by magnesium if haematin ester is used.

Aluminium. As the iron is tervalent in haematin and no other compound of the pigment containing a tervalent element was available, attempts were made to prepare a chromium or aluminium compound. All methods on similar lines to those adopted in the preparation of the other pigments failed and at present a chromium compound has not been produced. It was found however that by treating haematoporphyrin with pyridine saturated with anhydrous aluminium chloride at 60° a new pigment containing aluminium was produced. An examination of its properties shows that it must be regarded as the normal compound of the pigment with aluminium. It has a brilliant magenta colour and closely resembles the magnesium compound in its spectrum and shows a similar dusky yellow fluorescence. It is very soluble in both water and organic solvents, but the solution on standing gradually decomposes with the production of brown resinous material. Like the tin compound described by Milroy [1909], its most remarkable property is its stability to acids. The metal is removed from the magnesium compound by the weakest acids, but the aluminium compound can withstand the action of concentrated sulphuric acid, and of hydrobromic in acetic acid. The solution in concentrated sulphuric acid has a blue colour which only shows a broad region of absorption in the red, but on dilution with water the original colour and absorption spectrum return. The tin compound, like acid haematoporphyrin, dissolves in concentrated sulphuric acid without change of spectrum. When arsenic trichloride is substituted for aluminium chloride under the above conditions a solution of a deep yellowish-brown colour is produced. The spectrum resembles that of alkaline haematin; on pouring into alcohol a solution of acid haematoporphyrin is at once obtained showing that no permanent alteration in the pigment has taken place.

Alkali metals. Acid haematoporphyrin itself must be regarded as the hydrogen compound corresponding to the other metallic derivatives showing the characteristic two-banded spectrum. This is justified by an examination of the sodium and potassium compounds. The alkali metals can be introduced into haematoporphyrin by shaking a solution of the pigment in dry pyridine containing 5 % of methyl alcohol with an excess of powdered sodium or potassium hydroxide; the red-brown colour of the haematoporphyrin changes to a bright magenta in the case of the sodium and to red-purple in the case of the potassium. Traces of water or even alcohol added to the solution at once remove the alkali metal yielding ordinary alkaline haematoporphyrin.

By shaking a solution of alkaline haematoporphyrin with silver oxide or carbonate preferably in the presence of alcohol, silver is introduced and the solution shows a spectrum similar to that of the copper compound. Thus the univalent silver atom when introduced shows no similarity with the alkali metals. The stability of the silver compound to light and most reagents is of interest. Hydrochloric acid only slowly liberates haematoporphyrin, silver chloride being precipitated.

Cobalt. Of all the metallic derivatives of haematoporphyrin examined only those with iron and cobalt show a reduction and oxidation effect. Laidlaw [1904] found that the ordinary two-banded cobalt derivative was reduced to a one-banded form. By acting on a solution of the cobalt derivative in caustic soda with hydrazine hydrate it was reduced to a form showing two bands, one much stronger than the other and similar to those of the nickel compound. Thus this reduced form of the cobalt haematoporphyrin is probably the normal cobaltous compound corresponding to that of nickel. Oxidation to the original two-banded form took place with great ease, and the reduction could only be made to occur in strongly alkaline solution. By acting on the reduced form with nitric oxide a spectrum similar to that of the oxidised form was produced which did not change when treated with the reducing agent. As Laidlaw showed, there was no reaction with carbon monoxide. The oxidised form of the cobalt derivative shows little resemblance to oxyhaemoglobin. The substance is unaffected by hydrochloric acid and organic solvents, and the oxygen is not removed by a vacuum and only by the action of a powerful reducing agent. It seems clear that it involves a change from the cobaltous to cobaltic state and possibly more nearly resembles the oxidation of haemochromogen to haematin.

Collective properties of these compounds. When the absorption spectra of a series of metallic derivatives of haematoporphyrin are examined, those whose solutions have a red or purple colour can be placed in three groups which do not depend on the valency of the metal. For the type of each group we can take (1) acid haematoporphyrin, (2) oxyhaemoglobin and (3) haemochromogen. In group (1) the α band is less developed than the β band and the colour is usually purplish red or purple. In group (3) the α band is more strongly developed than the β band and the colour is usually scarlet red. Group (2) is intermediate in character. The main properties of metallic derivatives of haematoporphyrin are summarised in the following table. The figures refer to the above types of spectrum, whilst the letter H indicates a type of haematin spectrum. The property of dissolving in acetate buffer shows that the normal solubilities of the pigment are strongly influenced by combination with the metal. The ease with which the metal is removed by different acids is indicated by a positive or a negative sign.

			Metal removed by			
Metal	Type of spectrum	Soluble in acetate buffer	Water	Dilute acetic acid	Hydrochloric acid	Conc. sulphuric acid
Na	1	.	+	.	.	.
K	1	.	+	.	.	.
Ag	3	–	–	–	+	.
Mg	2	–	–	+	.	.
Zn	2	–	–	–	+	.
Al	2	+	–	–	–	–
Mn	H	+	–	–	–	+
Fe$^{\cdot\cdot}$	3	–	–	–	±	.
Fe$^{\cdot\cdot\cdot}$	H	–	–	–	–	+
Co$^{\cdot\cdot}$	3	.	–	.	.	.
Co$^{\cdot\cdot\cdot}$	2	+	–	–	–	+
Ni	3	–	–	–	–	+
Cu	3	–	–	–	–	+
As	H	.	+	.	.	.
Sn	2	+	–	–	–	–
Pb	H	.	–	+	.	.

2. THE ACTION OF STRONG ALKALI ON HAEMOGLOBIN.

In some unpublished observations J. S. Haldane found that when haemochromogen was prepared by adding caustic soda to reduced blood the mixture would, on standing, revert to haemoglobin. Experiments were tried in imitation of this, using different concentrations of alkali, and ferrous sodium tartrate as a reducing agent. In $N/2$ alkali the reduced haemoglobin shows a strong haemochromogen spectrum but the solution on dilution with water (being kept reduced) departs widely from Beer's absorption law, equivalent thicknesses, showing less of the haemochromogen spectrum as dilution proceeds until finally the absorption due to reduced haemoglobin becomes dominant. This clearly shows the reversible nature of the action of alkali.

When the solution of reduced haemoglobin in $N/2$ alkali is oxidised haematin results together with oxyhaemoglobin. It was found that, though the solution showed the haemochromogen bands only, about 50 % of the haemoglobin remained undecomposed. If, however, the solution in $N/2$ alkali was warmed it became of a lighter scarlet colour, and showed no alteration of absorption spectrum on dilution. When oxidised it yielded haematin alone—whether diluted or undiluted—showing a complete and irreversible decomposition into haemochromogen. Experiments were then tried using stronger solutions of alkali and all the solutions were cooled. As the experiments are only concerned with the *reversible* action of alkali, precautions had to be taken to avoid irreversible haemochromogen formation taking place. It was not found possible under the conditions used completely to eliminate this action, but it could be reduced to a low enough level not to interfere with qualitative experiments. It was found that minute traces of almost any foreign organic material--cork, wax, filter paper, or organic solvents in the soda solution—would cause an almost instantaneous irreversible haemochromogen formation, especially when the concentration of alkali added was high. In the absence of such impurities no such rapid action took place. Similarly hydrosulphite could not be used as a reducing agent; fortunately alkaline ferrous tartrate did not prove injurious even in a large excess.

The experiments were of the following type—a 1 % solution of haemoglobin either from washed corpuscles or defibrinated blood was reduced and cooled; to it was added a cooled solution of 40 % sodium hydroxide containing a trace of the reducing agent (ferrous tartrate) until a definite concentration of sodium hydroxide was reached; the mixture was then examined. At – 5° conversion into haemochromogen was not complete until the solution contained 20 % of sodium hydroxide. Such a solution had, when reversible, a scarlet colour, more brilliant than when the solution was irreversible; no difference has been detected up to the present in the bands, and on oxidation both are completely converted into alkaline haematin. The solution in both cases seems to contain ordinary haemochromogen. The change from reversibility to irreversibility must be due to a change in the protein—as is evidenced by a turbidity which often shows itself. To test the mixtures for reversibility they were diluted with an ice-cold solution of an ammonium salt (either sulphate or chloride) to which a little Stoke's reagent had been added. The soda is thereby replaced by ammonia which has comparatively little action on haemoglobin. Under these conditions the scarlet colour of the haemochromogen changed to the purple of haemoglobin, and the change was usually almost complete in 5 minutes. If irreversible haemochromogen formation had taken place the solution, instead of being clear, was turbid and remained scarlet indefinitely. The oxyhaemoglobin obtained after the alkali treatment had an absorption spectrum identical with that of the untreated substance, no shift in the bands being observable.

When the amount of alkali was increased so that there was 30 % in solution the pigment was completely precipitated as a scarlet flocculent mass, still showing the haemochromogen spectrum. The mixture could be converted into a clear solution of haemoglobin on treatment with an ammonium salt. If, however, it was allowed to warm to room temperature the precipitate swelled up slightly and became of a paler colour; it was then insoluble in the solution of an ammonium salt and gave no haemoglobin. When a solution of haemoglobin from twice washed corpuscles was used for the experiment the precipitate had a decidedly crystalline appearance and was intensely scarlet. A small quantity was filtered on asbestos in a Gooch crucible surrounded by an ice and salt jacket and washed twice with 40 % sodium hydroxide. The washings were colourless. On treating the residue with dilute ice-cold ammonium sulphate solution it partially dissolved forming a clear solution of haemoglobin. The insoluble portion consisted of coagulated protein and haemochromogen. From the homogeneous appearance of the precipitate obtained by the action of 30 % sodium hydroxide and from the fact that it will yield haemoglobin after washing with 40 % sodium hydroxide, it is concluded that it is a compound of haemochromogen and globin, probably of a colloidal nature. In solution in 20 % sodium hydroxide the haemochromogen cannot be precipitated in the presence of a calcium salt, whilst in the absence of globin no haemochromogen can remain in solution.

Thus, whilst the *specific* action of globin on haemochromogen is temporarily inhibited by alkali, the protein itself is capable of resisting the reagent. It now became important to examine the behaviour of oxyhaemoglobin and carboxyhaemoglobin under the same conditions. It is usually believed that oxyhaemoglobin yields haematin with moderate concentrations of alkali, but under the above conditions this is found not to be the case. Oxyhaemoglobin remained unaltered in colour and absorption spectrum in solutions of sodium hydroxide of concentrations up to 30 % and precipitation of the pigment did not occur. Carboxyhaemoglobin behaved similarly. When the solution of oxyhaemoglobin containing alkali was submitted to a vacuum both haematin and haemochromogen were formed, showing that the oxygen was still dissociable and that the haemoglobin, presumably formed, suffered decomposition by the alkali into haemochromogen which then became partially oxidised.

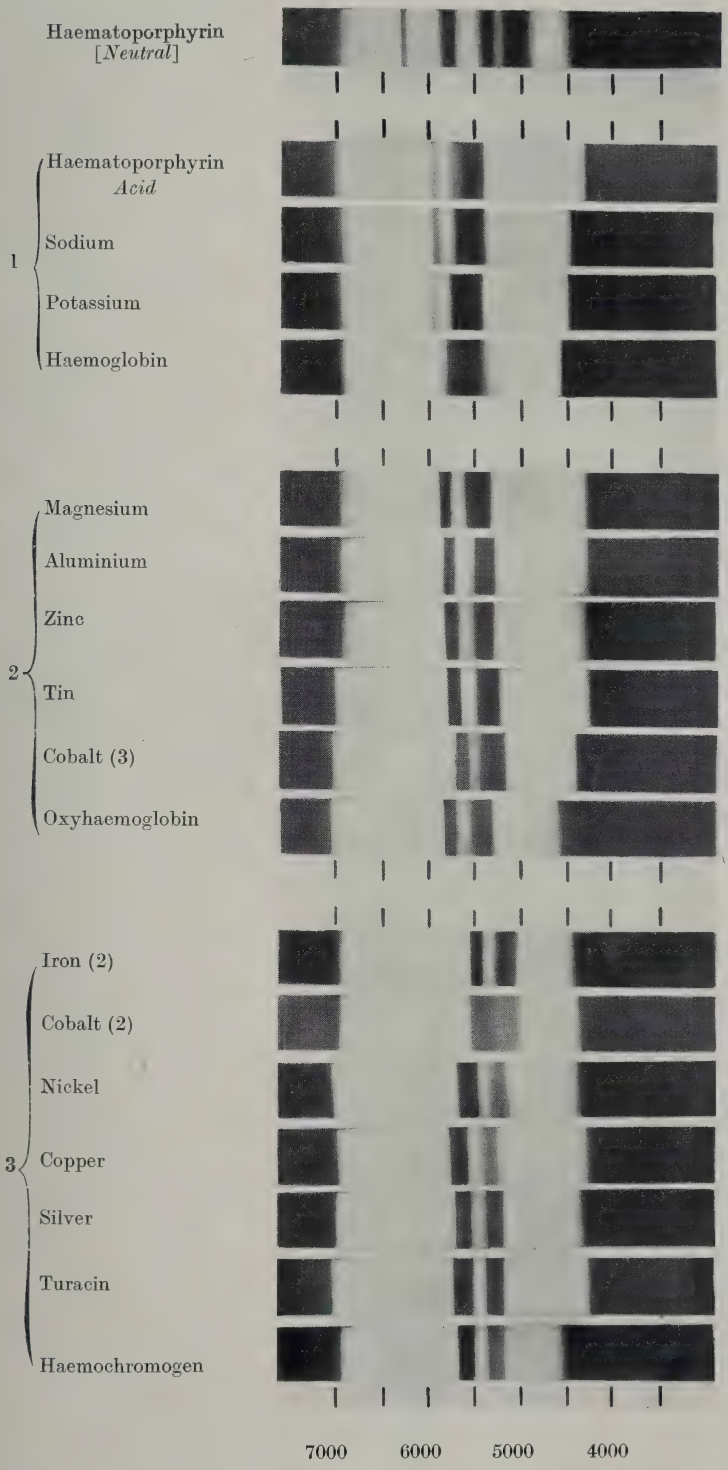
3. Haemochromogen in Pyridine.

A solution of haemochromogen in dry pyridine can readily be prepared. Whereas haemin dissolves in the moist solvent with a red-brown colour (giving the characteristic haematin-pyridine spectrum), in the dry solvent a brown solution is obtained having the absorption spectrum of acid haematin. This brown solution is completely reduced to haemochromogen on shaking for 1 minute with metallic mercury. Haemin thus resembles ferric chloride in the ease with which a chlorine atom is removed when dissolved in a dry organic solvent, the iron being reduced to the ferrous state. If haemin is added to dry piperidine, haemochromogen is at once produced without the addition of mercury.

The solution of haemochromogen in dry pyridine was bright scarlet and stable in air, not forming haematin as in the case of the aqueous solutions. Dry oxygen was passed through this solution at room temperature for 1 hour, and no change was observed either in the colour of the solution or in its absorption spectrum. The resistance to oxidation was diminished as increasing amounts of water were added. If an equal volume of distilled water was added the haemochromogen was completely converted into haematin. Coal gas passed through the solution produced no change in the absorption spectrum. Thus both the oxidation of haemochromogen to haematin and the formation of the carbon monoxide compound are prevented by pyridine, and if there is an oxygen compound analogous to that with carbon monoxide it might be thought to play a part in the oxidation of haemochromogen to haematin.

4. Methyl ester of Haemochromogen.

Amorphous haematin ester was obtained by washing an ethereal solution of haematin, methylated with dimethyl sulphate, with sodium carbonate solution; after drying with anhydrous sodium sulphate the solid ester was obtained by distilling off the ether. The dried product was dissolved in absolute alcohol and treated with the minimum quantity of hydrazine hydrate in an atmosphere of coal gas. Immediate reduction and rapid conversion of the haemochromogen ester into the carbon monoxide compound took place. The solution was warmed to 60° and diluted with water until precipitation of the pigment commenced. After cooling the solid was filtered off in an atmosphere of coal gas and washed with distilled water saturated with coal gas and containing a trace of hydrazine hydrate. It was dried in coal gas, all contact with air being avoided until the moisture had all been removed. The compound consisted of a pure Bordeaux-red powder, made up of minute hexagonal plates. It is soluble in all the usual organic solvents forming brilliant carmine red solutions which decompose in a few seconds, forming dull-brown solutions of haematin ester. If the solvent is carefully dried the action is retarded, but oxygen and moisture from the air rapidly produce oxidation. The absorption spectrum agrees closely with that of carbon monoxide haemoglobin. When the carbon monoxide ester of haemochromogen was dried over P_2O_5 in coal gas and dissolved in dry xylene, a stable carmine solution was obtained if air was excluded. But on passing dry oxygen the solution at once turned brown and was found to contain haematin ester. Even when great precautions were taken to avoid the presence of moisture haematin was produced by the action


Haematoporphyrin
[*Neutral*]
Haematoporphyrin
Acid
Sodium
1
Potassium
Haemoglobin
Magnesium
Aluminium
Zinc
2
Tin
Cobalt (3)
Oxyhaemoglobin
Iron (2)
Cobalt (2)
Nickel
3
Copper
Silver
Turacin
Haemochromogen
7000
6000
5000
4000

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of oxygen. The solid ester compound could not be kept; even over P_2O_5 it was gradually converted into a black powder.

It was found that pyridine not only prevents the formation of the carbon monoxide compound of haemochromogen but also causes the decomposition of that substance. When a little of the dry carbon monoxide compound was added to dry pyridine, the substance dissolved with vigorous effervescence forming a scarlet solution having the absorption spectrum of haemochromogen.

A concentrated solution of oxyhaemoglobin was prepared by centrifuging blood and laking the corpuscles with ether. One drop of this was added to 2 cc. of dry pyridine in a test-tube. Protein was precipitated and in the precipitate bubbles of gas were entangled. On standing 1 minute the clear liquid was scarlet red and showed the absorption spectrum of haemochromogen. Methaemoglobin prepared with ferricyanide and dialysed did not produce a trace of haemochromogen when added to pyridine but simply the brown-red solution of haematin.

SUMMARY.

1. Metallic derivatives of haematoporphyrin showing two-banded spectra can be divided into three classes which are independent of the valency of the metal.

2. Haemoglobin is completely changed to a substance having the spectrum and properties of haemochromogen by high concentrations of alkali. In the cold the action is reversible.

3. Oxyhaemoglobin and carbon monoxide haemoglobin are unaltered by high concentrations of alkali in the cold.

4. Haemochromogen in dry pyridine is not acted on by free oxygen, neither is a carbon monoxide compound formed.

5. Haemochromogen ester in organic solvents shows the characteristic reactions of the free substance.

6. Pyridine causes the decomposition of the carbon monoxide compound into free haemochromogen ester. Pyridine forms haemochromogen from oxyhaemoglobin as well as from haemoglobin.

I wish to thank Sir F. G. Hopkins for his kind encouragement and advice, and Dr Haldane for his private communication. I desire also to express my gratitude to the Department of Scientific and Industrial Research for grants held during the period of this research.

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LII. THE SPECIFIC ACTION OF PLANT FERMENTS.

I. THE SPECIFIC CONDITIONS OF ACTION OF LEAF INVERTASES.

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The investigations of Sörensen [1909], Michaelis and Davidsohn [1911] and Euler and Emberg [1919] on the optimal p_H-values for the action of yeast-invertases, and those of Euler and Svanberg [1921] for the action of intestinal invertase of man, gave a distinct dissimilarity of these values: the greatest action of yeast-invertase was found at p_H 3·5–5·5; that of intestinal invertase at p_H 5–7. That the invertases from various yeasts are not identical is made clear by Willstätter and Kuhn [1923].

Our experiments on the invertases of leaves of various plants give a new confirmation for the Willstätter point of view, according to which we have as many invertases as sources from which they are obtained.

We investigated the water extracts from air-dried powdered leaves of 14 species of wild and cultivated plants. The leaves were taken under identical conditions from various individuals of the same species (July–August, 1924), dried rapidly at 25–30° and ground in a mortar. 1 g. of a leaf-powder was mixed with 60 cc. of water and filtered through a dry filter after standing at 25° during 24 hours with toluene. 5 cc. of clear filtrate were mixed with 10 cc. of phosphate buffer mixture and 5 cc. 8 % saccharose solution. To all experimental flasks toluene was added and they were kept at 25°. The determinations of invert-sugar were made by the Benedict-Lewis picric acid method. The standard solutions were prepared each week according to Willaman and Davison's [1924] directions, with intervals of 0·125 mg. of invert-sugar. For each determination 0·5 cc. of the solution to be investigated was taken. The duration of each single experimental series varied from 23 to 91 hours at about 30°.

Exp. 1. The leaves of *Prangos pabularia* Lindl., a species of the Umbelliferae indigenous to the valleys of West Tian-Shan, were investigated at the Botanical Station of the University of Chimgan. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 1·0 mg.; duration of hydrolysis $47\frac{1}{2}$ hours.

p_H	Reducing sugar mg. in 0·5 cc.	Change	
		mg.	%
4·5	3·50	+2·50	48
5·0	4·75	3·75	71
5·6	5·25	4·25	81
5·9	5·50	4·50	86
6·2	5·75	4·75	90
6·5	6·00	5·00	95
6·6	6·25	5·25	100
7·0	5·25	4·25	81

Exp. 2. The subterranean leaves of bulbs of *Ungernia Severzovii* (Rgl.) B. Fedtsch., a species of the Amaryllidaceae from West Tian-Shan. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 1·125 mg.; duration of hydrolysis 49½ hours.

p_H	Reducing sugar mg.	Change	
		mg.	%
4·5	1·425	0·300	91
5·0	1·450	0·325	100
5·6	1·425	0·300	91
5·9	1·300	0·125	39
6·2	1·250	0·100	33
6·5	1·150	0·025	9
6·6	1·125	0·000	0

Exp. 3. The leaves of *Juglans fallax* Dode, from the Chimgan valley in West Tian-Shan. The initial content of reducing sugar is 0·650 mg.; duration of hydrolysis 46½ hours.

p_H	Reducing sugar mg.	Change	
		mg.	%
4·5	3·750	3·100	55
5·0	4·250	3·600	64
5·6	5·250	4·600	82
5·9	6·250	5·600	100
6·2	5·750	5·100	91
6·5	5·000	4·350	78
6·6	3·750	3·100	55
7·0	2·750	2·100	38

Exp. 4. The leaves of *Vitis vinifera* L. cultivated at Tashkent. The initial content of reducing sugar in 0·5 cc. of the experimental solution is 0·525 mg.; duration of hydrolysis 48 hours.

p_H	Reducing sugar mg.	Change	
		mg.	%
4·4	1·150	0·625	64
4·5	1·450	0·925	95
5·0	1·500	0·975	100
5·6	1·375	0·850	87
5·9	1·375	0·850	87
6·2	1·225	0·700	72
6·5	1·100	0·575	59
6·6	0·475	0·475	49
7·0	0·525	0·000	0

Exp. 5. The leaves of *Gossypium herbaceum* L. from the Turkestan Plant Breeding Station at Tashkent. The initial content of reducing sugar is 0·375 mg.; duration of hydrolysis 41 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·0	0·650	0·275	31
4·4	0·800	0·425	49
4·5	1·150	0·775	89
5·0	1·150	0·775	89
5·6	1·250	0·875	100
5·9	1·175	0·800	91
6·2	1·050	0·675	77
6·5	0·975	0·600	69
6·6	0·750	0·375	43
7·0	0·800	0·425	49

Exp. 6. The leaves of *Gossypium hirsutum* L. from the Turkestan Breeding Station at Tashkent (strains No. 182 and No. 509). The initial content of reducing sugar for No. 182 is 0·875 mg. and for No. 509 1·100 mg.; duration of hydrolysis for No. 182 46½ hours and for No. 509 45 hours 50 minutes. For p_H 4·0 and 4·4 glycine buffer solutions were used.

	No. 182			No. 509		
	Reducing sugar	Change		Reducing sugar	Change	
p_H	mg.	mg.	%	mg.	mg.	%
4·0	0·875	0·0	0	1·100	0·0	0
4·4	0·875	0·0	0	1·100	0·0	0
4·5	1·150	0·275	79	1·600	0·500	91
5·0	1·225	0·350	100	1·650	0·550	100
5·6	1·200	0·325	93	1·500	0·400	73
5·9	1·175	0·300	86	1·450	0·350	64
6·2	1·125	0·250	71	1·375	0·275	50
6·5	1·100	0·225	64	1·250	0·150	27
6·6	1·000	0·125	36	1·175	0·075	18
7·0	0·950	0·075	22	1·225	0·125	23

Exp. 7. The leaves of *Glycyrrhiza glabra* L. from Chimgan. The initial content of reducing sugar is 0·500 mg.; duration of hydrolysis 91 hours.

	Reducing sugars	Change	
p_H	mg.	mg.	%
4·0	0·500	0·0	0
4·4	0·500	0·0	0
4·5	0·900	0·400	100
5·0	0·850	0·350	88
5·6	0·775	0·275	69
5·9	0·600	0·100	25
6·2	0·525	0·025	6
6·5	0·500	0·0	0

Exp. 8. The leaves of *Platanus orientalis* L. from the University Botanical Garden at Tashkent. The initial content of reducing sugar is 0·625 mg.; duration of hydrolysis 47 hours 45 minutes. For p_H values from 3·3–4·0 glycine buffer solutions were used.

	Reducing sugar	Change	
p_H	mg.	mg.	%
3·3	0·925	0·300	34
3·7	1·250	0·625	72
4·0	1·250	0·625	72
4·5	1·500	0·875	100
5·0	1·425	0·800	92
5·6	1·375	0·750	86
6·2	1·250	0·625	72
6·6	0·900	0·275	32
7·0	0·875	0·250	29

Exp. 9. The leaves of *Pyrus communis* L. (Tashkent). The initial content of reducing sugar is 0·550 mg.; duration of hydrolysis 39 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	2·250	1·700	60
5·0	2·375	1·825	65
5·6	2·750	2·200	78
5·9	3·200	2·650	94
6·2	3·375	2·825	100
6·5	3·000	2·450	87
6·6	1·825	1·275	45
7·0	1·425	0·875	31

Exp. 10. The leaves of *Pyrus malus* L. (Tashkent). The initial content of reducing sugar is 0·825 mg.; duration of hydrolysis 49½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·4	4·750	3·925	72
4·5	5·875	5·050	93
5·0	6·160	5·325	98
5·6	6·250	5·425	100
5·9	5·950	5·125	95
6·2	5·500	4·675	86
6·5	4·625	3·800	70
6·6	3·500	2·675	49
7·0	2·625	1·800	33

Exp. 11. The leaves of *Quercus pedunculata* L. (Tashkent). The initial content of reducing sugar is 0·775 mg.; duration of hydrolysis 23 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	5·250	4·475	88
5·0	5·375	4·600	90
5·6	5·500	4·725	93
5·9	5·625	4·850	95
6·2	5·750	4·975	98
6·5	5·875	5·100	100
6·6	5·375	4·600	90
7·0	2·625	1·850	58

Exp. 12. The leaves of *Syringa vulgaris* L. (Tashkent). The initial content of reducing sugar is 0·675 mg.; duration of hydrolysis 38½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	1·075	0·400	89
5·0	1·025	0·350	76
5·6	1·025	0·350	76
5·9	1·050	0·375	83
6·2	1·125	0·450	100
6·5	1·050	0·375	83
6·6	1·075	0·400	89
7·0	0·950	0·275	61

Exp. 13. The leaves of *Fraxinus* sp. (Tashkent). The initial content of reducing sugar is 0·350 mg.; duration of hydrolysis 62 hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	1·450	1·100	92
5·0	1·475	1·125	94
5·6	1·500	1·150	96
5·9	1·500	1·150	96
6·2	1·550	1·200	100
6·5	1·500	1·150	96
6·6	1·300	0·950	79
7·0	1·200	0·850	71

Exp. 14. Finally, we investigated the expressed juice of *Salicornia herbacea* L. (from the salt desert Golodnaja Steppe). No reducing sugar at start; duration of hydrolysis 64½ hours.

	Reducing sugar	Change	
p_H	mg.	mg.	%
4·5	0·950	0·950	68
5·9	1·200	1·200	86
6·2	1·400	1·400	100
6·5	1·250	1·250	90
6·6	1·175	1·175	84
6·8	1·125	1·125	80
7·0	1·025	1·025	73
7·2	0·975	0·975	70
7·4	0·875	0·875	63
7·7	0·925	0·925	66
7·9	0·800	0·800	57
8·0	0·625	0·625	45

All our experiments give a definite conclusion that a definite optimal hydrogen ion concentration exists for each plant species and that variation of ratio of hydrolysis with change in p_H is of a very specific character.

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LIII. THE SPECIFIC ACTION OF PLANT FERMENTS.

II. THE SPECIFIC CONDITIONS OF ACTION OF LEAF PEPTASES.

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The investigations of Blagoveschenski and Sossiedov [1925] on the specific conditions of action of leaf invertases of various plant species have shown that each of these has a specific optimal p_H-value for its action. In the present paper we communicate our experiments on the action of plant peptases at various hydrogen ion concentrations.

We took samples of dried and finely ground leaves (exactly 1 g.) and mixed these with 10 cc. of 8 % peptone solution, 10 cc. of buffer (phosphate or glycine) mixture and some drops of toluene. All the flasks (two for each p_H-value) were kept in the thermostat at 25° or 30° for 72 hours. Each experimental series was followed by a control series without ferment action. The hydrolysis of peptone was measured by an amino nitrogen determination by Sörensen's method. The phosphates were removed with barium chloride and barium hydroxide. The formaldehyde titration was carried out with a comparator, for the elimination of the colour of the leaf extracts. The leaves were collected partly at Chimgan Botanical Station (*Astragalus Sieversianus* Pall., *Ungernia Severzovii* (Rgl.) B. Fedtsch., *Juglans fallax* Dode, *Prangos pabularia* Lindl., *Glycyrrhiza glabra* L.), partly in Tashkent Botanical Garden (*Syringa vulgaris* L., *Vitis vinifera* L., *Pyrus malus* L., *Pyrus communis* L., *Fraxinus* sp., *Quercus pedunculata* L., *Platanus orientalis* L.). The leaves of *Gossypium herbaceum* L. and *Gossypium hirsutum* L. were collected at the fields of the Turkestan Plant Breeding Station at Tashkent.

The data given in Table I demonstrate that the optimal concentrations of hydrogen ions for the action of peptases from leaves of various plants are very varied and specific for single plant species. It is interesting that at p_H 7·1, the neutral point, we could not find an optimum for any peptase, although they occur at both higher and lower p_H values.

We made a special comparative quantitative determination of the peptase action of 11 species at their optimal p_H values (Table II).

The activity of peptase (or its amount) in various plants is also specific.

Table I.

	p_H-values	4·5	5·4	5·8	6·3	6·7	7·1	7·7	8·0
		Change of amino nitrogen content.							
1. Ungernia	mg.	—	+0·7	2·1	3·5	3·8	3·8	5·3	6·3
Severzovii	%	—	11	33	56	60	60	83	**100**
2. Astragalus	mg.	—	4·9	1·8	4·9	6·7	9·1	11·9	8·4
Sieversianus	%	—	41	15	41	56	76	**100**	71
3. Syringa	mg.	8·4	4·2	5·6	8·4	7·0	14·0	15·4	14·0
vulgaris	%	55	27	36	55	45	91	**100**	91
4. Syringa	mg.	5·6	4·2	4·2	1·4	4·2	7·7	9·8	8·4
vulgaris	%	55	43	43	14	43	78	**100**	87
(middle)	%	55	35	39	35	44	85	**100**	89
5. Vitis	mg.	5·6	3·5	4·2	7·0	9·8	9·8	11·2	9·8
vinifera	%	50	32	37	63	88	88	**100**	88
6. Juglans	mg.	9·8	10·2	7·0	9·8	11·2	8·4	11·2	7·8
fallax	%	88	91	63	88	**100**	75	**100**	88
7. Prangos	mg.	—	4·9	5·9	—	9·8	8·4	9·1	7·4
pabularia	%	—	50	60	—	**100**	82	92	76
8. Glycyrrhiza	mg.	8·6	—	6·7	10·7	7·9	9·3	7·9	4·4
glabra	%	80	—	63	**100**	74	87	74	41
9. Pyrus	mg.	7·7	7·7	8·4	7·0	5·6	5·6	2·8	2·8
communis	%	92	92	**100**	89	67	67	34	34
10. Fraxinus	mg.	5·6	5·6	7·0	4·2	2·8	4·2	1·4	1·4
sp.	%	80	80	**100**	65	40	65	20	20
11. Gossypium	mg.	11·2	11·2	14·0	11·9	11·6	9·1	9·6	5·6
herbaceum	%	80	80	**100**	85	83	65	69	40
12. Gossypium	mg.	10·4	11·6	14·0	8·4	7·7	7·8	7·0	5·6
hirsutum, No. 182	%	74	83	**100**	60	55	56	50	40
13. Gossypium	mg.	9·8	11·8	13·3	10·5	8·4	6·3	7·0	4·2
hirsutum, No. 509	%	74	84	**100**	78	70	47	53	32
14. Quercus	mg.	23·8	—	14·0	11·2	8·4	9·8	9·8	11·2
pedunculata	%	**100**	—	59	47	35	41	41	47
15. Platanus	mg.	91	7·7	5·6	1·4	1·4	1·4	0·0	0·0
orientalis	%	**100**	85	62	15	15	15	0	0
16. Pyrus	mg.	7·0	—	5·6	—	2·8	1·4	0	0
malus	%	**100**	—	80	—	40	20	0	0

Table II.

Name	p_H (optimal)	Amino N change in mg.
Pyrus malus	4·5	+9·1
Pyrus communis	5·8	12·6
Vitis vinifera	7·7	14·0
Juglans fallax	6·7	12·8
Gossypium herbaceum ...	5·8	14·7
Gossypium hirsutum, No. 182	5·8	12·6
Gossypium hirsutum, No. 509	5·8	12·0
Syringa vulgaris	7·7	7·0
Fraxinus sp.	5·8	14·0
Platanus orientalis	4·5	7·0
Quercus pedunculata ...	4·5	14·0

All these results give a new confirmation to the point of view that physiologically plants can have just as definite properties as morphologically, and we have the right to suppose from these and many other similar observations and experiments the existence of a connection between the variability of the properties of living matter and that of external form.

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LIV. THE PHYSIOLOGY OF GASTRIC UREASE.

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(*Received March 12th, 1925.*)

We have recently shown that very active preparations of urease may be obtained from the stomachs of carnivora [Luck, 1924] and that the enzyme by its behaviour *in vitro* appears to be identical with soy bean urease [Luck and Seth, 1924].

As it was possible, in the absence of evidence to the contrary, that the enzyme might be a product of *post mortem* changes it was considered advisable to demonstrate if possible its presence *in vivo*. In proceeding to do so we have assumed that the observable effects would be either a catalysis of the hydrolysis of urea or the synthesis of that substance from ammonium carbonate. By this assumption we have not overlooked a view which theoretically and teleologically is more desirable. This is that both the synthetic and hydrolysing functions may be exercised by the enzyme, the direction of the reaction being controlled by the many physico-chemical factors which influence the equilibria within the cell.

Experimentally we have investigated the effect of altering the concentrations of urea and ammonium carbonate. In the one case the concentration of urea in the cells of the mucosa was raised by the gastric absorption of urea, or, as in one experiment, by the intravenous injection of that substance. The resulting effect on the cellular concentration of ammonia was observed by estimating at suitable intervals the ammonia content of the gastric venous blood. In the second case ammonium salts in aqueous solution were introduced into the stomach and urea estimations were made upon the blood flowing from that organ. In two experiments a third factor, the hydrogen ion concentration of the cell, was varied. This was done on the strength of that once popular idea that the formation of urea would be favoured by alkalosis and that of ammonia by acidosis.

The results suggest that the enzyme functions *in vivo* in the same way that it does *in vitro*. An increased concentration of urea in the cells of the gastric mucosa was invariably accompanied by an increase in the ammonia content of the gastric venous blood. On the other hand, we were unable to observe any indication of the gastric formation of urea under the conditions of our experiments.

EXPERIMENTAL.

All but the preliminary observations were made upon dogs which had fasted for 48 hours before use, to ensure freedom of the stomach from food. The animal was anaesthetised with ether and a tracheal cannula was inserted. The right femoral artery was exposed and a mid-ventral incision was made through the abdominal wall. The stomach was closed distally by a pyloric ligature. The mesenteric vessels were tied and the splenic vessels were ligatured close to the spleen. By this procedure the contents of the vena lienalis were limited to the blood which entered from the left gastro-epiploic vein, and the blood of the portal vein consisted principally of that from the stomach.

The blood samples of 6 cc. were drawn by syringe and were received in tubes containing 0·6 cc. of ammonia-free 2 % potassium oxalate. The syringe was washed with freshly distilled ammonia-free water before the drawing of a sample. The arterial punctures were made distal to the profunda artery. The gastric venous blood was drawn from the portal vein close to the porta hepatis. In general, six samples in all were collected, three portal and three arterial—the first pair serving as controls. After drawing the first pair of samples the experimental fluid was administered at 38°. The second and third pairs were drawn at approximately 1½ and 2½ hours after administration of the fluid. In some of the experiments modifications of this procedure were necessary. Where this has been so the experimental details have been given elsewhere in the text.

As we assumed the presence of a very labile ammonia-forming substance in blood [Medwedew, 1911; Henriques and Gottlieb, 1924; Parnas and Heller, 1924] the following method of ammonia estimation was designed to reduce autolytic changes to a minimum and to permit the simultaneous collection of several samples of blood. The principle of the method was the removal of the blood proteins with tungstic acid and the estimation of the ammonia in the centrifugate by distillation.

To 5 cc. of the oxalated blood were added immediately after collection 5 cc. each of ammonia-free water, 10 % sodium tungstate and 0·066 N sulphuric acid. The mixture was centrifuged. The centrifugates, which measured 11–13 cc. and were water-clear, were decanted into tubes and kept at − 2 to 0° until removed for estimation.

The method of distillation was designed to minimise secondary ammonia formation by hydrolysis and to permit direct nesslerisation of low volume distillates. Stanford's apparatus [Stanford, 1923] was used. By immersing both the receiver and the trap in crushed ice and evacuating with a Geryk pump, rapid distillation could be obtained with a bath temperature of 25–30°. 5 cc. of 0·05 N sulphuric acid were placed in the receiver and a similar volume in the trap; 10 cc. of the centrifugate were placed in the distillation flask. When the apparatus was sufficiently evacuated 5 cc. of alkali (consisting of

4 cc. of saturated sodium carbonate and 1 cc. of saturated sodium bicarbonate) were added. Distillation was continued for 30–40 minutes, in which time 8–9 cc. of distillate were collected. A second fraction obtained by continued distillation into a fresh receiver gave no colour with Nessler's reagent. Recovery of the ammonia was therefore complete. The contents of the receiver were weighed, nesslerised with one-tenth their volume of the reagent and compared with ammonium sulphate standards.

In the estimation of blood urea the tungstic acid precipitation of Folin and Wu [1919] was employed for the removal of proteins. To 4 cc. of the filtrate were added 0·3 cc. of a molar phosphate solution of p_H 7·45 and 1 cc. of 0·5 % urease [Van Slyke and Cullen, 1914] filtered through glass wool. After standing for 30 minutes at 40° the ammonia was distilled into standard acid and estimated by nesslerisation.

All tubes, pipettes, and other pieces of apparatus were repeatedly washed with freshly prepared ammonia-free water which was obtained by the distillation of dilute sulphuric acid and was used for the preparation of all reagents. The potassium oxalate was thrice recrystallised from ammonia-free water. The sodium carbonate solution was well boiled and when cold was added to the saturated solution of sodium bicarbonate. The sulphuric acid was obtained from freshly opened supplies and was found to contain only a very small quantity of ammonia. The final quality of the reagents was controlled by blank estimations.

The ammonia content of whole blood in vitro *at* 0°.

The necessity of immediate treatment of the blood samples is shown by the rapid increase in the ammonia content of rabbit blood even at 0°. A rabbit under ether anaesthesia was bled by cannula from the abdominal aorta. 35 cc. of blood were run into 5 cc. of the oxalate solution. 5 cc. of the oxalated blood were precipitated immediately. The remainder was stored at – 2 to 0° and precipitated in 5 cc. portions at varying intervals. The ammonia content of each centrifugate was estimated immediately.

Time after collection of blood	15 min.	1 h. 5 min.	2 h.	4 h. 5 min.	7 h.	24 h.
Mg. ammonia N per 100 cc. of blood	0·22	0·31	0·43	0·57	0·68	0·81

The ammonia content of the tungstic acid centrifugates at 0°.

By immediate precipitation of the blood samples it was found that the centrifugates could be stored at 0° without any increase in the ammonia content. It was concluded that either the labile ammonia precursor was precipitated by the tungstic acid or the acidity of the centrifugate inhibited ammonia formation. 30 cc. of blood were drawn from the abdominal aorta of a rabbit and run into 5 cc. of oxalate. Six portions of 5 cc. each were immediately precipitated and centrifuged. The centrifugates were stored at – 2 to 0°.

Time after precipitation	25 min.	1 h. 40 min.	2 h. 35 min.	5 h. 40 min.	23 h. 30 min.
Mg. ammonia N per 100 cc. blood	0·11	0·08	0·09	0·11	0·10

The recovery of added ammonia.

40 cc. of rabbit arterial blood were collected in 5 cc. oxalate. To separate portions of 10 cc. each were added respectively, 10 cc. of water, 10 cc. aqueous solutions of ammonium sulphate. Sodium tungstate and sulphuric acid were added in the usual manner. The ammonia contents of the centrifugates were estimated by two different methods (*a*) by distillation, (*b*) by the Nash-Benedict apparatus [1921].

Mg. ammonia N per 100 cc. blood.

Total ammonia found		Ammonia added	Ammonia recovered	
(*a*)	(*b*)		(*a*)	(*b*)
0·05	0·04	—	—	—
0·11	0·10	0·054	0·06	0·06
0·25	0·23	0·22	0·20	0·19
0·43	0·43	0·44	0·38	0·39

From these experiments it was concluded that if the blood samples were precipitated immediately after collection the ammonia content of the centrifugates could be estimated at leisure on the same day and that the values obtained would represent with sufficient accuracy the ammonia content of the shed blood.

The formation of ammonia in shed blood.

Having confirmed the findings of Parnas and Heller on the rapid formation of ammonia in rabbit blood *in vitro*, we were interested in the results obtained by Nash and Benedict [1921]. The evidence advanced by these authors indicated that the ammonia content of the shed blood of dogs did not increase during a post-collection period of several hours. On repeating these experiments we found the very marked specific differences shown in Fig. 1. For a comparison of methods, parallel estimations on the same blood were made

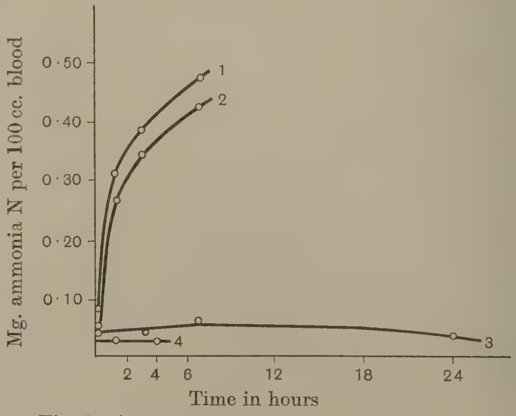

Fig. 1. Ammonia formation in blood at 2 to 0°.

1. Rabbit blood—Distillation method.
2. Rabbit blood—Nash-Benedict method.
3. Dog blood—Nash-Benedict Method.
4. Dog blood—Distillation method.

by our method and by that of Nash and Benedict. In each case the animal was bled from an artery during ether anaesthesia. The blood was collected in oxalate and was kept at – 2 to 0°.

The differences in behaviour between rabbit and dog blood are so striking as to suggest that the latter does not contain a labile ammonia-forming substance or that some necessary enzyme is absent. Incidentally the results also show that by the application of two very different methods of estimation essentially the same values for the ammonia content of the blood have been obtained. This indicates either that the two methods give accurate and absolute values or that both are subject to a common error. Of the two possibilities the latter is the more improbable.

Experiments with urea.

In Exps. 1–5 which follow we have studied the effect of an increased urea concentration in the cells of the gastric mucosa on the ammonia content of the gastric venous blood. The results have been summarised in Table I.

From these experiments it was concluded that if the urea concentration in the cells of the gastric mucosa be increased, either by the gastric absorption of urea or by diffusion from the blood, part of the urea is converted into an ammonium salt.

The administration of the urea in dilute hydrochloric acid and the positive results following intravenous injection of urea show that the observed effects cannot be due to the action of ureolytic organisms which might be in the stomach. The original experiments, which were all done *in vitro* in the presence of toluene, and in which it was found that the hydrolysis of urea was effected only by preparations of the gastric mucosa, also prove that the enzyme is a constituent of the gastric cells in the carnivora.

The results of two control experiments are presented in Table II.

The results presented in Table II, Exp. 6, proved that for the increased ammonia production in the gastric mucosa, as recorded in Table I, the presence of urea was necessary. In Exp. 7 the ammonia content of the mesenteric blood prior to the urea absorption was already so high that the results are little more than suggestive. The absence of any increase in the ammonia content, and in fact its very noticeable decrease, do at least suggest that the intestinal absorption of urea is not accompanied by an increased ammonia production. This view is supported by the results of experimentation *in vitro* by which it was shown that urea is not hydrolysed by preparations of the intestinal mucosa [Luck, 1924]. One should also refer to the persistently low values of the arterial ammonia, for they indicate the absence of any general systemic production of ammonia.

It might therefore be concluded that under the conditions of these experiments urea was converted into ammonia by the gastric mucosa only, and that this hydrolysis of urea was catalysed by gastric urease.

Table I.

	Mg. N per 100 cc. blood.				
	Portal vein		Femoral artery		
Time	NH_3	Urea	NH_3	Urea	Remarks
Exp. 1. Male dog of 12 kg.					
10.30	0·17	—	—	—	
10.36	—	—	0·08	—	
10.40	—	—	—	—	3 g. urea in 50 cc. water administered by stomach tube
11.12	—	—	0·10	—	
11.30	0·27	—	—	—	
12.10	0·52	—	—	—	
12.15	—	—	0·12	—	
Exp. 2. Male dog of 23 kg.					
11.00	—	—	—	7·5	
11.04	0·13	5·9	—	—	
11.07	—	—	—	—	5 g. urea in 100 cc. water administered by stomach tube
11.35	—	—	0·12	8·8	
11.40	0·39	10·0	—	—	
12.41	—	—	0·22	11·0	
12.43	1·60	14·2	—	—	
12.50	—	—	—	—	Stomach excised. Urease present in mucosa
Exp. 3. Female dog of 9 kg. Animal pregnant. Uterus excised.					
10.42	—	—	0·24	5·6	
10.46	0·25	5·8	—	—	
10.50	—	—	—	—	3·7 g. urea in 75 cc. 0·05 *N* HCl by stomach tube
11.42	0·54	13·6	—	—	
11.45	—	—	0·21	12·3	
Exp. 4. Male dog of 13 kg.					
10.55	—	—	0·07	12·5	
11.00	0·07	12·3	—	—	
11.04	—	—	—	—	5 g. urea in 100 cc. 0·05 *N* HCl by stomach tube
11.47	—	—	—	17·8	
11.50	0·12	17·0	—	—	
1.04	—	—	0·05	18·1	
1.06	0·28	26·2	—	—	
Exp. 5. Male dog of 15 kg.					
10.31	—	—	0·07	—	
10.40	0·13	—	—	—	
10.50–11.10	—	—	—	—	4 g. urea in 100 cc. 0·085 % NaCl injected in the left femoral vein
11.50	0·14	—	—	—	
12.53	—	—	0·11	—	
12.56	0·32	—	—	—	
1.10	—	—	—	—	Stomach excised. Urease present in the mucosa

(Note to Exp. 5.—To induce acidosis, the animal received on each of the two fasting days 4·8 g. anhydrous calcium chloride in milk.)

Experiments with ammonium salts.

Though we have shown that gastric urease functions *in vivo* by the hydrolysis of urea it is also apparent that it may be capable of urea synthesis. In Exps. 8 and 9 we have followed the urea content of arterial and gastric venous blood during the gastric absorption of ammonium salts.

Table II.

Time	Mg. ammonia N per 100 cc. blood.		Remarks
	Portal vein	Femoral artery	
Exp. 6. Male dog of 13 kg.			
10.52	—	0·19	
10.55	0·18	—	
11.00	—	—	100 cc. 0·05 *N* HCl by stomach tube
12.04	0·15	—	
12.56	—	0·14	
1.03	0·18	—	
Exp. 7. Female dog of 18 kg.			
10.20	—	0·02	
10·30	1·39	—	
10·45	—	—	4 g. urea in 100 cc. 0·05 *N* HCl were injected into a loop of the small intestine (jejunum and ileum)
11.23	—	0·06	
11.28	0·49	—	
12.26	0·46	—	

(Note to Exp. 7.—The spleen was tied off and the splenic vein was ligatured close to the portal vein. The mesenteric vessels were not tied.)

Table III.

Time	Mg. N per 100 cc. blood.							
	Portal v.		L. gastro-epiploic v.		Femoral a.		Femoral v.	
	NH_3	Urea	NH_3	Urea	NH_3	Urea	NH_3	Urea
Exp. 8. Male dog of 12 kg.								
10.30	—	—	0·20	8·5	—	—	—	—
11.05	—	—	9·8	8·2	—	—	—	—
11.13	—	—	—	—	—	—	—	10·6
11.16	—	—	—	—	2·10	—	—	—
12.03	—	—	2·0	14·9	—	—	—	—
12.09	—	—	—	—	—	—	0·98	13·1
12.10	—	—	—	—	1·02	17·3	—	—
12.15	2·29	16·7	—	—	—	—	—	—
12.47	—	—	3·2	19·4	—	—	—	—
12.52	—	—	—	—	—	—	1·1	12·8
12.54	—	—	—	—	1·01	17·9	—	—
12.57	3·4	19·4	—	—	—	—	—	—
(Note.—10.34: 5 g. ammonium chloride in 100 cc. water by stomach tube.)								
Exp. 9. Male dog of 11 kg.								
10.59	—	—	—	—	0·11	—	—	—
11.02	0·21	13·4	—	—	—	—	—	—
11.50	—	—	—	—	0·36	19·4	—	—
11.55	3·9	19·2	—	—	—	—	—	—
12.43	—	—	—	—	0·35	23·1	—	—
12.48	2·5	21·5	—	—	—	—	—	—

(Note.—On each of the two fasting days the animal received 10 g. sodium bicarbonate in milk. 11.08: 4 g. ammonium carbonate in 100 cc. water by stomach tube.)

As the urea content of the blood leaving the stomach is practically the same as that of the inflowing arterial blood, there is no indication of any gastric synthesis of urea from the absorbed ammonia. The available evidence therefore favours the view that gastric urease functions *in vivo* by the conversion of urea into ammonium carbonate and that to no measurable degree does it manifest any synthetic function.

DISCUSSION.

As a first consideration it is apparent that gastric urease cannot be associated with the production of the urinary or blood ammonia. Any ammonia which it may produce under the most favourable conditions would be promptly converted into urea by the liver. Even if some general significance could be attached to the portal ammonia, most of it enters from the mesenteric veins and not from the gastric veins.

As a second conclusion, it would therefore follow that gastric urease could have only a local function if any. This consideration limits the function of the enzyme to the production of ammonia for secretion into the stomach or in response to some local intracellular requirement. Without becoming involved in too much speculation it is sufficient at present to enumerate the various possible functions of the enzyme that have been suggested to us, viz., the secretion of alkali into the stomach for the control of gastric hyperacidity [McBaird, Campbell and Hern, 1924; Huber, 1917], an intracellular mechanism for the protection of the cells of the mucosa against the highly acid gastric juice with which they are in contact, a mechanism for the local regulation of the movements of the stomach wall either by the direct effect of the base (ammonium carbonate) on the muscle fibres [Kupaloff, 1924; Goldenberg, 1924] or as a specific property of ammonium ions [Prawdicz-Neminski, 1924]. A final suggestion would be to class the enzyme as a vestigial structure. Without the support of further experimental evidence we are not prepared however to ascribe any specific function to the enzyme.

SUMMARY.

(1) Under normal physiological conditions urea constantly diffuses into those cells of the gastric mucosa which contain urease and is converted into ammonia. In dogs under ether anaesthesia an increased diffusion of urea caused either by the gastric absorption or intravenous injection of that substance is accompanied by an increase in the ammonia content of the gastric venous blood.

(2) The gastric absorption of ammonium salts is not accompanied by an increase in the urea content of the gastric venous blood.

(3) The enzyme appears to function only by the conversion of urea into ammonia in response to local conditions or requirements.

(4) The shed blood of dogs shows no increased ammonia content in 24 hours at − 2 to 0°. That of rabbits rapidly increases.

We would conclude by expressing our indebtedness to Sir F. G. Hopkins for much helpful criticism, to Dr J. B. S. Haldane for many valuable suggestions, and to the Medical Research Council for the assistance that has been extended to one of us (J. M. L.).

Note. Since preparing these results for publication an interesting paper by Parnas [1925] has appeared in which are reported the blood ammonia values for several different species. His observations on the specific differences in the behaviour of rabbit and dog blood after long standing are in entire agreement with the results which we have recorded.

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LV. THE RELATION BETWEEN THE METABOLISM AND THE SPECIFIC DYNAMIC ACTION OF AMINO-ACIDS.

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In a series of papers on "Animal Calorimetry," by Lusk and his collaborators [1912–1924], the view has been put forward that the specific dynamic action of proteins is due to a chemical stimulation of the body cells to a higher level of metabolism by some intermediary metabolites of the amino-acids other than the immediate products of their deamination. Accordingly one would expect, *a priori*, the amino-acids glycine and alanine—which exert a profound specific dynamic action—to be metabolised in the organism more rapidly and more completely than leucine, aspartic acid, etc., which are known to exert no appreciable specific dynamic action [Lusk, 1915; Atkinson and Lusk, 1918]. In other words, one would expect a smaller increase in the amino-N content of blood and a quicker return of the same to the normal level, following the ingestion of glycine or alanine, than after the ingestion of an equivalent amount of leucine or aspartic acid.

However, contrary to expectations based on Lusk's theory, the observations of Bang [1916, 2] show that whereas the amino-acid content of rabbit's blood increases very rapidly after an oral administration of glycine or alanine, and remains at a high level for about 6 hours, the ingestion of leucine causes no significant increase at all in the amino-acid content of blood.

In view of the importance and interest of the problem and the apparent inconsistencies existing between Lusk's theory and Bang's observations, it was decided to repeat Bang's experiments on a more extended scale. The results of this part of the investigation are described in Part I.

Further, having recently been impressed by the undesirability of applying facts based on observations on one species of animals to other species, we extended Bang's experiments to dogs in order to make sure that the vastly different points of view arising from the work of Lusk and Bang were not due to some intrinsic metabolic differences in the species of animals used by them. The modified procedure adopted in these experiments was designed firstly to eliminate any effect due to variable times of passage of the ingested material through the pylorus, and secondly to enable us to follow the course of intestinal absorption more closely by analysing blood samples from the portal vein. This part of the investigation is dealt with in Part II.

A third series of experiments in which the amino-acids were administered to dogs by intravenous injection is described in Part III. These experiments were designed for the purpose of eliminating any effect due to variable rates of absorption from the intestine.

EXPERIMENTAL.

PART I

Rabbits of approximately uniform size were employed, each animal being used not more frequently than once a fortnight, during which interval it was kept on the ordinary routine diet. For 48 hours before the experiment the rabbit was kept in a clean metabolism cage and was given only water. On the experimental day, blood samples of about 1·5 cc. each were obtained from the ear at suitable intervals, and received into 0·15 cc. ammonia-free 2 % potassium oxalate solution. After drawing the first sample of blood a neutralised solution, at 38°, of the substance under investigation was administered by stomach tube. The total volume of the fluid ingested was 100 cc., and contained 560 mg. of amino-N (equivalent to 3 g. glycine).

Immediately after drawing each sample, the blood proteins were precipitated by the method of Folin and Wu [1919]. For the estimation of urea, 0·3 cc. molar phosphate solution of p_{H} 7·45, and 1 cc. of 0·5 % urease [Van Slyke and Cullen, 1914] filtered through glass-wool, were added to 4 cc. of the protein-free blood filtrate, and, after standing for 45 minutes at 40°, the ammonia was distilled (using 2 cc. saturated borax solution) into standard acid, and estimated by nesslerisation.

The amino-N was estimated in the blood filtrate by Folin's colorimetric method [Folin, 1923] which yielded very consistent and satisfactory results.

The water and other reagents used were ammonia-free, and all apparatus was washed with such water.

The amino-acids investigated were glycine, alanine, leucine, aspartic acid, glutamic acid, cystine, histidine and tryptophan, of which only the first three were used by Bang [1916, 2].

Results.

Table I shows the changes in the urea content of rabbit's blood during a period of about 7 hours following the ingestion of the various amino-acids, and Fig 1[1] represents the corresponding changes in the amino-acid content of the blood, under the same conditions.

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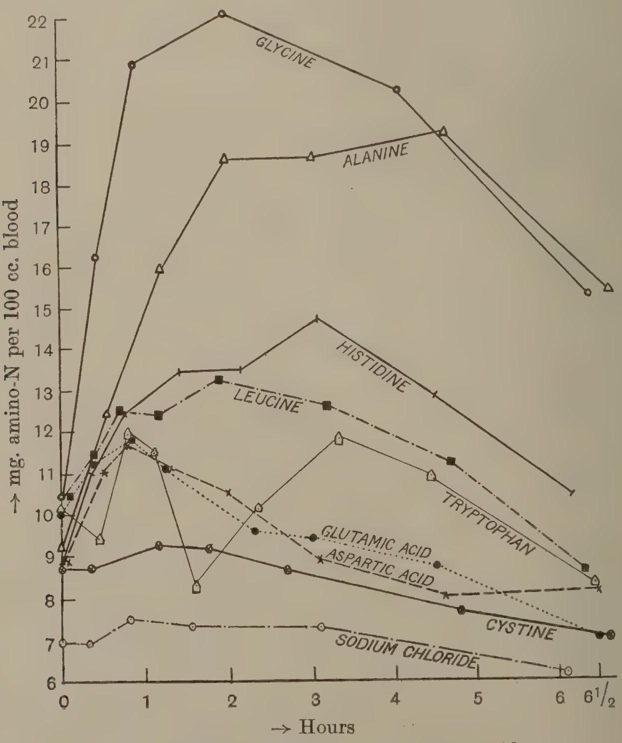
[1] In the case of tryptophan the amino-N content of blood was reckoned as the difference between the total-non-protein N and urea-N, and the figures thus obtained being rather high, only two-thirds of their actual values were plotted in Fig. 1 for the sake of a clearer comparison.

Table I. *The Urea-values in this Table are expressed in mg. urea-N per* 100 *g. blood.*

Substance under investigation	Period after ingestion of amino-acid							Difference between initial and maximum urea values	Remarks
	0 min.	20–25 mins.	45–50 mins.	75–90 mins.	2¼–3 hrs.	4–4½ hrs.	6–6¾ hrs.		
Glutamic acid (6·0 g.)	9·6	10·3	10·9	12·6	14·7	14·8	18·6	9·0	
Glycine (3·0 g.)	18·0	—	19·6	17·6	19·1	22·2	26·7	8·7	
Aspartic acid (5·3 g.)	13·6	13·3	15·8	17·2	19·9	20·1	21·7	8·1	
l-Alanine (Kahlbaum) (3·6 g.)	15·8	—	—	18·8	23·4	23·4	16·4	7·6	
Cystine (4·8 g.)	12·4	12·4	—	17·9	18·7	14·0	15·1	6·3	Administered as the sodium salt
Tryptophan (4·5 g.)	14·3	16·1	16·6	18·3	20·0	19·4	18·2	5·7	Animal had acute diarrhoea about 1 hour after ingestion of tryptophan
l-Leucine (Kahlbaum) (5·2 g.)	14·5	14·4	15·7	18·3	17·5	17·5	18·8	4·3	Administered as the sodium salt
Histidine hydrochloride (8·3 g.)	21·6	22·9	22·3	22·0	19·3	21·2	21·7	1·3	
Histidine hydrochloride (repeated) (8·3 g.)	18·9	20·1	20·0	20·1	18·9	18·4	19·0	1·2	
Sodium chloride (1·2 g.)	10·7	8·5	8·8	9·6	10·1	—	6·1	–0·6	Control experiment



(Period after the ingestion of the amino-acid)
Fig. 1. Amino-N content of rabbit's blood.

These results (Table I) indicate that whereas there is no marked difference in the extent of urea formation in the case of glycine, alanine, aspartic acid and glutamic acid, there is a progressive decrease in the case of cystine, tryptophan, leucine and histidine, the last one producing practically no increase in the blood urea during the entire experimental period of 6 hours. On the other hand, the amino-acid content of blood (Fig. 1) after the ingestion of glycine, alanine or histidine not only attains a very high maximum, but persists at a high level all through the experimental period; whereas the amino-acid content of blood in the case of the other amino-acids examined, without ever rising very high, comes down to the normal or subnormal level within 3–6 hours, in confirmation of Bang's observations [1916, 2]. The significance of these results is discussed elsewhere.

PART II

Having in the main confirmed Bang's observations on rabbits regarding the amino-acid content of blood after the ingestion of glycine, alanine and leucine, and having further extended the investigation to other amino-acids, the experiments were repeated on dogs, in the following way:

Dogs weighing about 10 kg. each were used, and they were given water only for 48 hours before use to ensure freedom of the small intestine from food. The animals were anaesthetised with ether[1]. A tracheal cannula was inserted. The femoral artery on one side and the femoral vein on the other were exposed. The abdomen was then opened and the splenic vein was tied off close to the portal vein. Ligatures were then applied to the small intestine, one just below the duodenum and the other above the caecum. The femoral artery was then ligatured distal to the profunda artery. Blood samples were taken by syringe at suitable intervals from the femoral artery and vein and from the portal vein, the latter being punctured about 2–3 inches from the porta hepatis. After drawing the first set of blood samples, the substance under investigation in a neutralised solution[2] at 38°, was injected into the small intestine by means of a hypodermic needle and pipette. The abdomen was promptly closed with artery clips. The total volume of the fluid injected was 100 cc. The animal was kept warm throughout the experiment.

The collection of blood samples (about 1·5 cc. each), the removal of blood proteins and the estimation of urea and amino-N in the blood filtrates, were done exactly as described before (p. 367).

The amino-acids investigated were glycine, alanine, leucine, aspartic acid, glutamic acid and cystine, the amounts used being the same as in the experiments on rabbits (see Table I).

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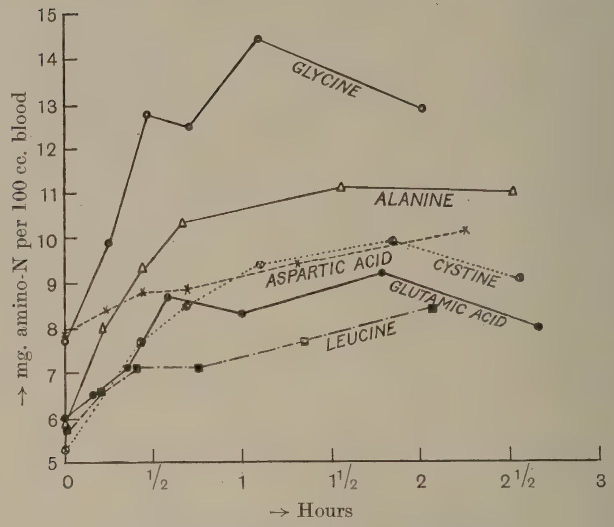
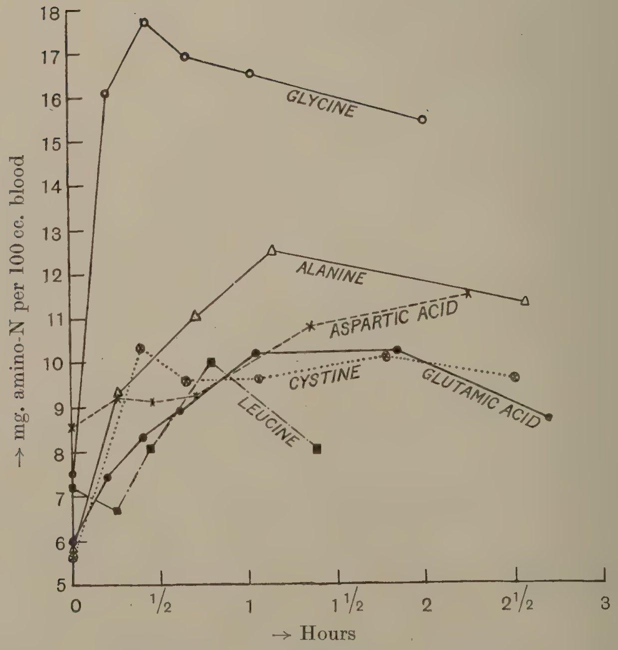
[1] Ether anaesthesia has, according to Okada and Hayashi [1922] no significant effect on the amino-acid content of dog's blood, during the experimental period of about 2 hours.

[2] Cystine was administered as the sodium salt, whilst the leucine solution was neutralised just before injection.



(Period after the ingestion of the amino-acid)

Fig. 2. Amino-N content of dog's arterial blood.



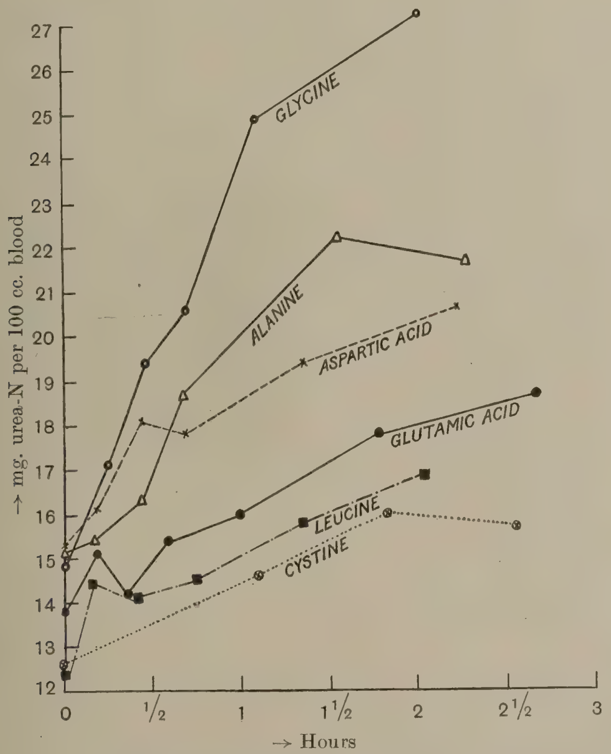
(Period after the ingestion of the amino-acid)

Fig. 3. Amino-N content of dog's portal blood.

Results.

(i) In confirmation of the observations made on rabbits (see Part I), Figs. 2 and 3 show that the amino-acid content of blood after the ingestion of glycine or alanine not only attains a very high maximum but persists at a high level all through the experimental period, whereas in the case of the other amino-acids studied, the amino-N content remains at a comparatively low level throughout. The possible significance of this observation is discussed later.

(ii) The extent of urea formation in the case of glycine and alanine is higher than in the other cases (Fig. 4).



(Period after the ingestion of the amino-acid)

Fig 4. Urea content of dog's arterial blood.

(iii) A comparison of Figs. 2 and 3 shows that at any one time the amino-acid value of portal blood is higher than the corresponding arterial value. This presumably results from the dilution of the portal blood with systemic blood. In the early stages of the experiment, the amino-N content of femoral-venous-blood[1] is also lower than that of the corresponding arterial sample. Later on, the two values tend to be almost identical. These observations, supported by the results of our injection experiments (Part III), point to a rapid absorption of the amino-acids from blood by the tissues. These conclusions are in agreement with similar findings of Folin and Denis [1912, 1] and of Van Slyke and Meyer [1912].

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[1] The figures for amino-N of femoral-venous blood were obtained in the case of glycine, alanine and cystine only, but they are not included in Fig. 2, in order to avoid unnecessary complication of the curves.

PART III

Dogs weighing about 10 kg. each were used. After being given only water for 48 hours, the animals were anaesthetised as described in Part II. The femoral artery on one side and the femoral vein on the other were exposed. The bladder was emptied by pressure. The femoral artery was then ligatured distal to the profunda artery. After drawing the first arterial blood sample by syringe, as described in Part II, the amino-acid under investigation, dissolved in 0·85 % NaCl solution, neutralised to litmus and warmed to 38°, was injected into the femoral vein as rapidly as possible (usually in less than a minute). Subsequent arterial samples were drawn at suitable intervals and analysed for amino-N and urea, as described in Part II. The total volume of the fluid injected was 25 cc., and it contained 186·6 mg. of amino-N. The amino-acids investigated were glycine, alanine, and aspartic acid.

At the end of the experimental period, the bladder was emptied and washed by catheterisation, the urine being subsequently analysed for amino-N.

Table II. 1·0 *g. glycine injected intravenously.*

Intervals before or after injection	mg. N per 100 cc. blood	
	Amino-N	Urea-N
Before injection	5·9	7·7
2 mins. after injection	10·0	7·9
8 ,, ,,	8·4	7·8
17 ,, ,,	6·7	8·7
28 ,, ,,	6·5	8·2
38 ,, ,,	6·3	8·7
57 ,, ,,	5·8	7·6
69 ,, ,,	5·8	7·7
94 ,, ,,	5·9	8·3
122 ,, ,,	5·4	8·5

Essentially similar results were obtained with alanine and aspartic acid.

These results (Table II) show that:

(i) Immediately after injection, the amino-acid content of the blood falls very rapidly, reaching almost the normal level within 15 minutes.

(ii) During the experimental period of 2 hours, there is no significant increase in the blood urea.

No measurable amount of amino-N was eliminated in the urine during the course of the experiment.

From these observations it is concluded that, under the conditions of our experiments, the tissues absorb the amino-acids almost completely and with extreme rapidity from the blood, and that the formation of urea follows much later.

Discussion.

We have shown (Parts I and II) that the extent of deamination of glycine and alanine is, in the case of rabbits of the same order, and in the case of dogs even greater than that of the other amino-acids studied. Again our injection experiments (Part III) have shown that under similar conditions the rate of absorption of the different amino-acids by the tissues is of a similar order, and also that the excretion of the free amino-acid is negligible under the conditions of our experiments. From these considerations it is clear that the earlier and greater increase in the blood amino-N, and its persistent circulation at a high level following the ingestion of glycine or alanine (both in rabbits and in dogs), is due neither to the lack of deamination nor to the non-absorption of these amino-acids by the tissues.

It is suggested that this phenomenon is due to the rapid absorption of glycine and alanine from the intestinal tract, as shown by the early and rapid rise in the amino-N of the portal blood, and its persistence at a high level for a long time (Fig. 3). Levene and Kober [1909] and Levene and Meyer [1909] have shown that glycine, alanine, glycyl-glycine and aspartic acid are rapidly metabolised, whilst leucine, phenylalanine, arginine and asparagine are metabolised more slowly. They ascribe these differences to the different rates of absorption of these amino-acids from the alimentary canal. Further, the work of Bang [1916, 1] and of Lewis [1918], on the relation between the amount of amino-acid ingested and the rise in the amino-N content of blood, lends additional support to the view expressed above.

Other contributory factors leading to this phenomenon of the rapid and continuous entrance into the systemic circulation of glycine and alanine (as distinct from other amino-acids), may be firstly, the permeability of the liver cells to glycine and the non-permeability to leucine, as observed by Bang [1916, 2], and secondly, the greater adsorbability of glycine and alanine by the blood corpuscles as suggested by the work of Sbarsky and Muchamedoff [1925] on the adsorption of glycine, alanine, leucine and tyrosine by an "erythrocyte-emulsion."

From the above-stated considerations it is obvious that Bang's observations [1916, 2] regarding the persistent circulation in blood of amino-N at a high level, following the ingestion of glycine or alanine, are not necessarily out of harmony with the views of Lusk on the cause of the specific dynamic action of these amino-acids. These two apparently inconsistent points of view are reconciled and explained by the difference in the rates at which the various amino-acids enter the circulation from the digestive tract.

It may be mentioned that the greater urea production in the case of glycine and alanine (Part II) is also probably a direct result of the circulation of larger amounts of these amino-acids and their consequent higher "partial pressure" in the sense of Folin and Denis [1912, 2].

The cause of the specific dynamic action of amino-acids.

Lusk [1915] considered that the increased heat production following the ingestion of glycine or alanine is not due to the oxidation of these amino-acids or of their metabolites, but that it is caused by the chemical stimulation of the body cells to a higher level of metabolism. In their attempts to discover the nature of this stimulant and the mechanism of its action, Lusk and his associates came to the following conclusions:

(i) That the specific dynamic action of amino-acids bears no relationship to the rate or extent of deamination, as shown by the facts that the period of maximum heat production [Lusk, 1912; Csonka, 1915] precedes the period of maximum nitrogen excretion [Janney, 1915] and that glutamic and aspartic acids and asparagine, though deaminised, exert no specific dynamic action [Atkinson and Lusk, 1918].

(ii) That acid radicals as such, or hydroxy- or keto-acids, which may possibly be the intermediary metabolites of some amino-acids, do not exert any specific dynamic action [Lusk, 1921; Atkinson and Lusk, 1918].

These results left the main problem still unsolved, namely, whether the specific dynamic action is due to a stimulus provided by the free amino-acid itself or by one or more of its yet unknown non-nitrogenous metabolites. Lusk [1915], however, subscribed to the latter view and the two chief arguments which he put forward in support of his theory need some attention.

Firstly, Lusk argued that if the free amino-acids exert any specific dynamic action, one should be able to observe an appreciable accumulation of amino-acids in the tissues following the ingestion of meat. Since Wishart [1915] failed to observe any such accumulation, Lusk concluded that the stimulus to increased heat production was not provided by the free amino-acids themselves. It appears to us, however, that this argument, besides being based on observations which are contradicted by Folin and Denis [1912, 2], Van Slyke [1914] and Mitchell [1918], is in itself not very conclusive. Our observations and those of numerous other workers have established the fact that following the ingestion of a protein or an amino-acid diet, the amino-N content of blood remains at a high level for a considerable period, in spite of the rapid absorption by the tissues. This fact indicates clearly that, under the conditions described, a fresh and continuous supply of amino-N reaches the tissues during a period of several hours. Therefore, even though there may not be a static accumulation of amino-acids in the tissues, there is no reason to assume that the abundant supply of constantly circulating amino-acids is not capable of stimulating the tissue cells to a higher level of metabolism.

The second argument put forward by Lusk in support of his theory is based mainly on the observations of Janney and Csonka. Janney [1915] showed that in phlorhizinised dogs the excretion of glucose, following the ingestion of a protein, was proportional to the amount of glucogenetic amino-acid radicals present in that protein, and that the glucose excretion attained a maximum value during the second and third hours. Csonka [1915] observed that in phlorhizinised dogs the period of maximum heat production (second and third hours) after the ingestion of meat or glycine, corresponds to the period of maximum metabolism of the ingested amino-acids, as determined by the "extra-glucose" excretion method. From these observations Lusk concluded that the specific dynamic action of amino-acids was dependent upon and proportional to their metabolism. However, this conclusion is not justified in view of the facts that glutamic and aspartic acids, in spite of their glucogenetic properties [Janney, 1915], do not exert any specific dynamic action [Atkinson and Lusk, 1918], whilst leucine and tyrosine, though non-glucogenetic [Janney, 1915], cause a certain increase in heat production [Lusk, 1915]. Again, alanine produces only about half as much "extra heat" as an iso-glucogenetic quantity of glycine [Lusk and Richie, 1915], though, according to the "glucogenesis" theory, the two should produce the same effect.

And finally, the correspondence in the periods of maximum heat production and maximum glucose excretion cannot be brought forward as an argument in support of Lusk's theory, because it has been shown by us (Fig. 1) and by Bang [1916, 2] that, under similar conditions, the amino-N content of blood is also at its maximum level during the second and third hours[1]. Hence the only conclusion that can be drawn from the above considerations is that the specific dynamic action may be due to some property of either the free amino-acid itself or its metabolites. The existing data do not preclude either of the two possibilities.

In this connection, however, the observations of Sandiford, Boothby and Giffin [1923], that "In all instances (of myelogenous leucaemia) in which amino-acids (of blood) were increased, there was some, but not necessarily a proportional increase in basal metabolism," are of interest. Similar observations on the association of pathological hyperamino-acidaemia with increased basal metabolism, have been recorded in cases of phosphorus poisoning [Atkinson and Lusk, 1918], and pernicious anaemia [Kraus, 1893; Magnus Levy, 1906; and Meyer and Du Bois, 1916]. But although an elevated basal metabolism, under physiological as well as pathological conditions, has been shown to be intimately associated with an increased concentration of amino-acids in the blood, it cannot be stated at this stage that the increased heat production is a direct consequence of hyperamino-acidaemia.

SUMMARY.

(1) Bang's observations on rabbits regarding the persistent circulation of amino-N in the blood at a high level, following the ingestion of glycine or alanine, and the absence of an appreciable increase in the amino-N content of the blood after the ingestion of leucine, are confirmed and extended to other amino-acids, using both rabbits and dogs.

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[1] In the experiments on dogs, the maximum level of amino-N in the blood is reached a little earlier (Fig. 2) owing to more rapid absorption from the small intestine.

(2) In the case of histidine, although a high and sustained level of amino-N in the blood is maintained, as in the case of glycine and alanine, yet there is no increase in the urea content of blood even after 6 hours. All the other amino-acids studied caused appreciable urea production.

(3) The specific dynamic action of an amino-acid is proportional to its power of increasing the amino-N content of the blood, following the gastro-intestinal absorption of the amino-acid.

(4) An explanation is offered for the apparent inconsistencies in the views of Lusk regarding the cause of the specific dynamic action of amino-acids and the above observations of Bang and ourselves.

(5) The cause of the specific dynamic action of amino-acids is discussed.

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LVI. ON THE METHODS OF FITTING THE FORMULA OF MICHAELIS IN RELATION TO THE EFFECT OF HYDROGEN ION CONCENTRATION ON ENZYME ACTION TO THE DATA: WITH SOME DISCUSSION OF THE RESULTS.

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I PROPOSE in this paper to discuss the theory of Michaelis regarding enzyme action and to give a note on the methods which I have found useful in fitting his theoretical curves to the experimental data. According to this theory, when an enzyme acts in solutions of different hydrogen ion concentrations only that portion of the enzyme which is not ionised retains its activity. The curve describing the rate of action of an enzyme is thus according to Michaelis: (1) "the undissociation curve of an acid," or (2) "the undissociation curve of a base," or (3) "the undissociation curve of an ampholyte." "We can therefore understand all the phenomena completely by the following interpretation: the enzyme represents an acid, a base, or an amphoteric electrolyte." Caution, however, may be needed in interpreting the experiments. With some enzymes which are not really ampholytes, the observations give a grouping which suggests this, because a strong acid or a strong alkali causes destruction of the enzyme, the diminution in action observed being due to this and not to an increased ionisation.

The physico-chemical theory and the equations formed by Michaelis are as follows. The dissociation of water into ions obeys the well-known equation:

$$H_2O \rightleftarrows H^{\cdot} + OH'$$

which is commonly written

$$[H^{\cdot}] \cdot [OH'] = k_w \quad \text{.................................(1)},$$

where k_w is defined as the dissociation constant of water and is equal to 10^{-14} at temperature 18°. As $[H^{\cdot}]$ is equal to $[OH']$, the number of hydrogen and hydroxyl ions present in a neutral solution is thus 10^{-7} g. ions per litre.

With an acid enzyme, the undissociated residue, *i.e.* the ratio of the undissociated acid to the total acid denoted by ζ is given by the equation

$$\zeta = \frac{1}{1 + \frac{k}{[H^{\cdot}]}} \quad \text{.................................(2)}.$$

For a base in a similar way

$$\zeta = \frac{1}{1 + \frac{k}{[\mathrm{OH}^{\cdot}]}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3).$$

Working with such small numbers as 10^{-7}, it is convenient to use the index to denote the hydrogen ion concentration. The symbol p_{H} which is equal to $-\log_{10} \mathrm{H}^{\cdot}$ has been selected for convenience, p_{H} being a contraction for power of the radix 10. If this substitution is made in equation (2), we have

$$\zeta = \frac{1}{1 + k\,10^{-p_{\mathrm{H}}}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(4),$$

or in ordinary coordinates,

$$y = \frac{1}{1 + k\,10^{-x}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(5).$$

In the case of the amphoteric electrolyte the equation becomes

$$\zeta = \frac{1}{1 + \frac{k_a}{[\mathrm{H}^{\cdot}]} + \frac{k_b}{k_w}[\mathrm{H}^{\cdot}]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6),$$

where k_a and k_b are the dissociation constants of the acid and the base. This equation describes a symmetrical curve. Referring to the axis of symmetry it becomes

$$\zeta = \frac{1}{1 + k\,(10^{\xi} + 10^{-\xi})} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7),$$

which is the convenient form for fitting. If ϕ is the distance of the axis of symmetry from the origin in (6)

$$k_a = k\,10^{\phi}$$

and

$$k_b = k_w\,k\,10^{-\phi}.$$

In the case of a dibasic acid

$$\zeta = \frac{1}{1 + \frac{k_1}{[\mathrm{H}^{\cdot}]} + \frac{k_1 k_2}{[\mathrm{H}^{\cdot}]^2}}$$

$$= \frac{1}{1 + k_1 10^{+x} + k_1 k_2 10^{+2x}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(8),$$

where k_1 signifies the first and k_2 the second dissociation constant of the acid.

In the case of an amphoteric electrolyte which is doubly dissociated both as regards the acid and basic radicals, a case not given by Michaelis

$$\zeta = \frac{1}{1 + k_1\,(10^{\xi} + 10^{-\xi}) + k_2\,(10^{+2\xi} + 10^{-2\xi})} \quad \ldots\ldots\ldots\ldots\ldots\ldots(9).$$

k_1 and k_2 are here constants such as k in equation (7).

Cor. It may happen that double dissociation is confined either to the acid radical or the basic in which case the term $10^{2\xi}$ or $10^{-2\xi}$ is omitted respectively.

A further case not considered by Michaelis is that in which the dissociation takes place from grouped molecules. In this case ξ is replaced by $c\xi$, and the curve of the amphoteric electrolyte, equation (7), becomes

$$\zeta = \frac{1}{1 + k\,(10^{c\xi} + 10^{-c\xi})} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(10).$$

A considerable number of enzymes have been examined and the agreement of the theory and experiment is in many cases very close. The lipase of the stomach may be taken as an example of an acid enzyme, and that of the pancreas of an alkaline, as shown by the experiments of Davidsohn [1913]. The examples of ampholytes are numerous and it is only necessary to refer to the experiments by Sörensen [1909] with catalase where observations and the theory are in very close accord. There are, however, many exceptions. The inversion of raffinose by the enzyme raffinase is one case. The simple theory no longer accounts for the phenomena observed. In the accompanying diagram (Diagram I), the observations of Kuriyama and Mendel [1917] have been graphed and two curves drawn through the observations. The chain line shows the curve of an ampholyte with one dissociation as given by Michaelis, the continuous line the curve with two dissociations. The observations are, as is obvious from the diagram, not of a high order of accuracy, but I think they are sufficiently accurate to show that the second curve accords more closely with the facts.

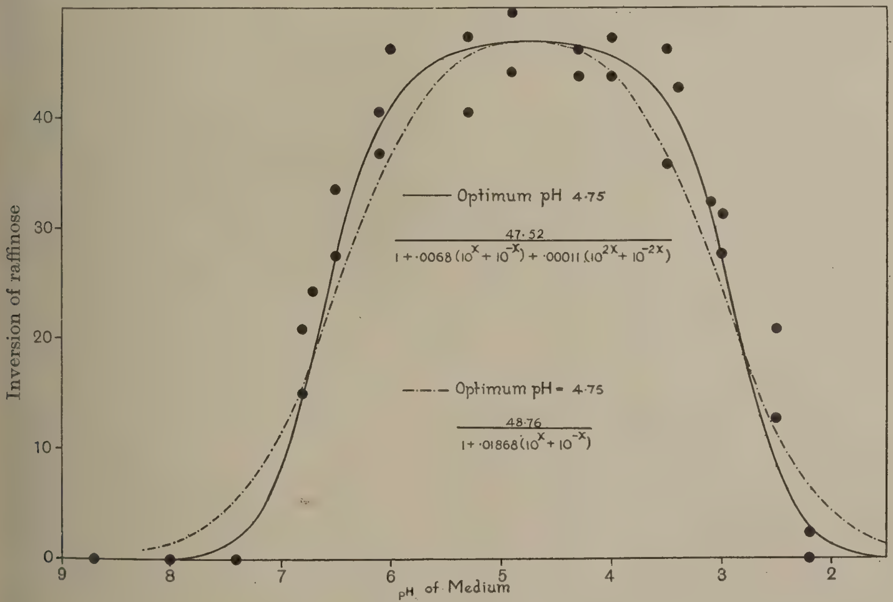

Diagram 1. Action curve of raffinose (Kuriyama and Mendel).

In testing any theory much is to be learnt from the exceptions to the theory. For instance, with regard to the observations on a certain enzyme, which observations I had permission given me to use, but which I do not wish to use at the present moment because the experiments are being repeated, the figures obtained were irreconcilable with the Michaelis theory as given but were reconcilable with that theory if the molecules of the enzyme existed on an average linked in pairs and if these pairs tended to lose one ion. In this case there may be an error but the figures are so definite that I think it unlikely that the repetition of the experiments will alter the conclusions. In other words the formula (10) applies, in this case the value of c being equal to 0·5. To certain other observations on enzymes, the same remarks apply, but the data are not sufficient to permit of an undoubted interpretation. Data, however, which are sufficient to settle this point may be obtained from the observations on lysins.

The experiments examined are those of Walbum [1915] who worked with various haemolysins and gives a very large number of tables and diagrams illustrating the phenomena. Since, however, both alkalies and acids in moderate concentrations lake red blood corpuscles, experiments with haemolysins often do not afford a sufficient range of values to permit of curve fitting. To measure the action of the acid or of the alkali separately and subtract this effect from the total effect is also of doubtful validity as it cannot be directly inferred that a mixture of lysin and acid or alkali produces only an additive effect. The effect of a lysin or an acid or alkali may be greater upon a slightly damaged, than on a normal red blood corpuscle.

One lysin, however, that of the poison of the bee, furnishes quite sufficient data. The bloods of three animals, the sheep, the ox, and the horse, were used. The observations on ox blood corpuscles fit a Michaelis curve having an optimum action with $p_H = 7 \cdot 6$ very well. A rather better fit is obtained on the assumption that two ions are dissociated. The observations, however, have a considerable probable error and the curves therefore cannot be statistically separated. With sheep corpuscles the dissociation of two ions is, however, quite definitely required. Both theories fail with the blood corpuscles of the horse. If the Michaelis theory holds here, only one explanation seems possible, namely, that from two linked molecules, an average of five ions is liberated. The possibility of this I leave to the chemists, but it seems to me improbable. In these observations the optimum value of the hydrogen ion concentration for the action of the lysin varies with the substrate. The difference of the optimum, passing from the sheep through the ox to the horse, is fully half a unit of concentration expressed as p_H.

The rate of the destruction of enzymes at different hydrogen ion concentrations follows similar laws. It is found that an enzyme is destroyed more rapidly by heat as the amount of ionisation increases according to the Michaelis theory. They add nothing to the general theory. With regard to toxins and lysins, however, there are some points of interest. Observations on the destruction of a staphylolysin have been made by Walbum [1922, 1] at three different temperatures and in a range of hydrogen ion concentrations from 4·5–9·5. The observations and the curves I have fitted are given in the accompanying table (Table I). The observations at 30° show an asymmetrical curve, the descent being slower on the alkaline side than on the acid. These are compatible with the hypothesis that there are two dissociations on the acid side and one on the alkaline. The optimum of permanence is with $p_H = 6$. Experiments at temperatures of 35° and 40° show a different phenomenon. The optimum concentration for survival is at the same point as before, but in this case the curves are symmetrical and the observations can only be fitted with two dissociations on both sides of the optimum. It is to be noted that with increase of temperature, the range of survival is continuously limited.

Table I. *Destruction of a staphylolysin at three different temperatures.*

30°			35°			40°		
	Amount in 1 cc.			Amount in 1 cc.			Amount in 1 cc.	
p_H	Act.	Th.	p_H	Act.	Th.	p_H	Act.	Th.
9·09	2·0	3·66	8·70	2·0	2·42	8·04	2·0	2·41
8·65	9·52	8·25	8·32	5·71	7·37	7·45	13·3	12·77
8·16	17·5	15·97	7·85	18·2	16·77	6·37	25·6	28·58
7·55	22·2	24·11	7·20	25·0	25·51	5·28	28·6	27·10
6·44	27·0	28·40	5·95	28·6	28·59	4·51	13·3	14·26
5·33	28·6	28·09	4·83	25·0	24·95	3·89	2·0	1·93
4·54	24·4	23·75	4·15	14·7	14·77	3·59	2·63	0·84
3·91	10·4	10·73	3·75	2·0	6·85			
3·61	5·26	4·54						

The same law also applies to the destruction of toxins (Diagram II). Walbum [1922, 2] gives details regarding the destruction of the diphtheria toxin with hydrogen ion concentration for two different years, namely, 1919 and 1920. The toxin prepared in 1919 shows a symmetrical curve requiring for a fit the dissociation of two ions on both sides. The curve for 1920 requires two dissociated ions on the acid side but only one on the alkaline. As the toxins were of equal efficacy this would seem to indicate that part of the complex of which the toxins are constituted is subject to variation and suggests that this part is not an essential constituent of the toxic group of atoms.

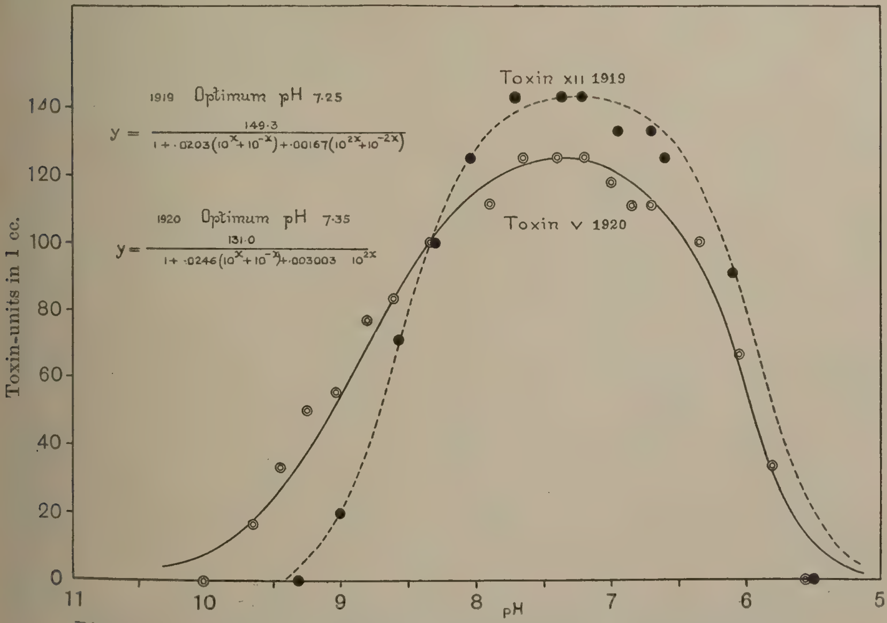

Diagram II. Destruction of diphtheria toxin at different p_H values (Walbum).

APPENDIX.

I. The first of the curves is that with the equation

$$y = \frac{a}{1 + k\,10^{-x}}.$$

The easiest way to fit this curve is to draw a free hand line through the observations and then taking two values of the ordinates at a distance of one unit of hydrogen ion concentration, to solve for the constants. If the two values be α and β, the solution is

$$k = \frac{\beta - \alpha}{10\alpha - \beta}.$$

The value for a is

$$a = \alpha\,(1 + k).$$

The middle point of the curve is at a distance $-\log k$ from the point of origin chosen. The rest of the curve may immediately be drawn by using the values of 10^x given in the table. Plotting the curve thus calculated through the observations the amount of readjustment can be guessed at once. By moving the middle point either to the right or to the left the process described may be repeated. No further approximation is usually required. The fit assumes that the maximum a is not known. If it be known one observation alone is sufficient to determine k.

Table II. *Table showing the values of the functions used in the calculations.*

x	10^x	10^{-x}	$10^x + 10^{-x}$	$10^{2x} + 10^{-2x}$
0	1·000000	1·0000	2·0000	2·0000
·25	1·778279	·5623	2·3406	3·4785
·50	3·162278	·3162	3·4785	10·1000
·75	5·623413	·1778	5·8012	31·6544
1·00	10·00000	·1000	10·1000	100·0100
1·25	17·78279	·0562	17·8390	316·2310
1·50	31·62278	·0316	31·6544	1000·0010
1·75	56·23413	·0178	56·2519	3162·2783
2·00	100·0000	·0100	100·0100	10000·0001
2·25	177·8279	·0056	177·8335	31622·7800
2·50	316·2278	·0032	316·2310	100000·0000
2·75	562·3413	·0017	562·3431	316227·8000
3·00	1000·000	·0010	1000·0010	1000000·0000
3·25	1778·279	·0006	1778·2796	
3·50	3162·278	·0003	3162·2783	
3·75	5623·413	·0002	5623·4132	
4·00	10000·00	·0001	10000·0001	
4·25	17782·79	·0001	17782·7901	
4·50	31622·78	·0000	31622·7800	
4·75	56234·13	·0000	56234·1300	
5·00	100000·0	·0000	100000·0000	
5·25	177827·9	·0000	177827·9000	
5·50	316227·8	·0000	316227·8000	
5·75	562341·3	·0000	562341·3000	
6·00	1000000·	·0000	1000000·0000	

II. The next curve is that referring to the amphoteric electrolyte:

$$y = \frac{a}{1 + k\,(10^x + 10^{-x})}.$$

Here it is best to determine from the observations the situation of the axis of symmetry. Having drawn a free hand curve through the graph of the data and then ruled a series of lines parallel to the axis of x, the middle points of these lines are determined with the help of a scale and then averaged. This average may be taken as the axis of symmetry. Only two ordinates are needed to determine a and k. If α and β be the two values chosen, α referring to the ordinate at the axis of symmetry and β to that at a distance of unit hydrogen ion concentration from it, we have at once

$$k = \frac{\alpha - \beta}{10{\cdot}1\beta - 2\alpha}$$

$$a = \alpha\,(1 + 2k).$$

The curve can at once be constructed by the use of the table $10^x + 10^{-x}$. As the curve is symmetrical only one side needs to be calculated. When the calculated curve has been fitted to the observations it is quite easy to see whether a further approximation is necessary or not.

III. Sometimes the data cannot be adjusted to the formula just given; such a case is the hydrolysis of raffinose. It is necessary then to proceed to the formula describing double dissociation, *i.e.*

$$y = \frac{a}{1 + k\,(10^x + 10^{-x}) + \lambda\,(10^{2x} + 10^{-2x})}.$$

The solution of this equation is obtained as in the last case by choosing the axis of symmetry, estimating its ordinate, also the ordinate at the distance of one unit of hydrogen ion concentration from it and that also at a distance of two units. The method of solution is simply to put the appropriate values of x and y in the equation and solve the three simultaneous equations for the three constants a, k and λ. If α, β and γ be the three values at the axis of symmetry and at a distance of one and two units of hydrogen ion concentration respectively, the formulae are as follows

$$a\left\{\frac{90998{\cdot}00091}{\alpha} - \frac{19799{\cdot}9802}{\beta} + \frac{179{\cdot}82}{\gamma}\right\} = 71377{\cdot}84071,$$

$$89{\cdot}91\lambda = \left(\frac{a}{\beta} - 1\right) - 5{\cdot}05\left(\frac{a}{\alpha} - 1\right),$$

$$\tfrac{1}{2}\left(\frac{a}{\alpha} - 1\right) = k + \lambda.$$

In this case k and λ are essentially positive, if either is found to be negative the formula cannot hold. The drawback to this method is that in calculating a, at least five decimals are required. This follows because in general $\frac{a}{\alpha}$ and $\frac{a}{\beta}$ will have both of the first figures unity with a zero following, and as in the formula unity is subtracted from these ratios, it is necessary to have four significant figures.

IV. In the case where there is unequal dissociation on either the acid or the alkaline side one term in the coefficient of λ disappears. Here the formula is

$$\frac{a}{1 + k\,(10^x + 10^{-x}) + \lambda\,10^{2x}}.$$

The equations for solution are then

$$a\left\{\frac{90999}{\alpha}-\frac{19899{\cdot}99}{\beta}+\frac{189{\cdot}9}{\gamma}\right\}=71288{\cdot}91,$$

$$100\left(\frac{a}{\alpha}-1\right)-\frac{a}{\beta}-1=189{\cdot}9\,k,$$

and

$$\frac{a}{\alpha}-2k-1=\lambda.$$

V. The last case to be considered is the one in which "linked" molecules occur. The equation is

$$y=\frac{a}{1+k\,(10^{cx}+10^{-cx})}.$$

The solution is really simple but in the process of elimination some unsuitable solutions are introduced. If 10^c be denoted by l, we have, choosing the axis of symmetry and taking three values at distances of unit hydrogen ion concentration, the following three equations

$$\alpha=\frac{a}{1+2k}\quad\ldots\ldots\ldots\ldots\ldots\ldots(1),$$

$$\beta=\frac{a}{1+k\,(l+l^{-1})}\quad\ldots\ldots\ldots\ldots\ldots\ldots(2),$$

$$\gamma=\frac{a}{1+k\,(l^2+l^{-2})}\quad\ldots\ldots\ldots\ldots\ldots\ldots(3).$$

These lead, after the inapplicable roots have been removed, to an equation for l of the form

$$l^2-\left\{\frac{\alpha\,(\beta-\gamma)}{\gamma\,(\alpha-\beta)}-1\right\}l+1=0\quad\ldots\ldots\ldots\ldots\ldots\ldots(4).$$

The roots of this equation have the values l and $\frac{1}{l}$. From equations (1) and (2), we have

$$k=\frac{\alpha-\beta}{p\beta-2\alpha},$$

where p denotes $l+\frac{1}{l}$ or the coefficient of l in equation (4). From equation (1), a is at once obtained. For interpolating values, between those observations originally chosen for fitting, if these are required for graphical purposes, it is usually sufficient to take the values of the square roots of l and $\frac{1}{l}$ from a table of square roots and the values of l^2 and l^{-2} from a table of squares, whence $l^{1{\cdot}5}$, $l^{2{\cdot}5}$, etc. can be immediately calculated.

With lysins the range of activity is often less than with enzymes, in which case it may be inconvenient to take the unit hydrogen ion concentration as the basis of measurement, half a unit or even less being more convenient. This does not change the solution given above but only the numerical interpretation.

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LVII. A STUDY OF THE OXIDATION OF THE AMMONIUM SALTS OF NORMAL SATURATED FATTY ACIDS AND ITS BIOLOGICAL SIGNIFICANCE.

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In a number of papers Dakin [1908, 1; 2, 3; 1910] described the production of β-keto-acids and the ketones arising from them by loss of carbon dioxide, in a series of experiments on the oxidation of the ammonium salts of normal saturated fatty acids with hydrogen peroxide. The reaction is a general one and at the time it was discovered was of great significance because it afforded great support to the evidence for the theory of β oxidation of fatty acids propounded by Knoop [1904]. It is a good instance of a process taking place *in vitro* which has helped to throw light on a similar process occurring in the living body. The reactions which occur when fatty acids and certain of their phenyl derivatives undergo oxidation in the body are not simple. Phenylpropionic acid has been shown by Dakin [1908, 4, 5] to yield, in addition to the corresponding β-keto-acid, the β-hydroxy-acid and the α, β-unsaturated acid. It seemed worth while therefore to find out whether Dakin's observations on the oxidation of fatty acids with hydrogen peroxide could be extended to include these other oxidation products, and especially the unsaturated acids which are sometimes formed during oxidation in living animals.

Using conditions similar to but not identical with those of Dakin, attempts were made in the first instance to find out whether stearic acid yielded unsaturated acids on oxidation.

At first it was thought that this was so, an acid giving a lead salt soluble in ether being isolated from the products of oxidation. This acid absorbed iodine, but not in amount sufficient to account for one double linkage, and in other ways was different from the unsaturated acids of the oleic series. The investigation of this substance has not been completed but in addition to this acid (or mixture of acids) another new acid was isolated which proved on further investigation to be γ-keto-stearic acid. This showed therefore that besides β oxidation, γ oxidation also takes place. The γ-keto-acid could not be isolated quantitatively so that it is not possible to compare accurately the extent of oxidation at the β and γ carbon atoms. The β-keto-acid, under the conditions of the oxidations, will decompose practically quantitatively to yield its corresponding ketone and this can be easily estimated. The amount obtained in several experiments indicated that β oxidation of the stearic acid had occurred to the extent of about 6 %. The amount of γ-keto-acid isolated corresponds to about 1 %, but this must be regarded as a minimum figure. In these experiments a large part of the stearic acid is recovered unchanged. It seems probable that other keto-acids are also produced. This certainly happens when the lower fatty acids are oxidised and will be referred to presently, but with the higher acids, their isolation from the large amount of unchanged fatty acid has not been achieved.

In order to see whether other homologues of stearic acid yielded a γ-keto-acid on oxidation, experiments were carried out on similar lines with palmitic and myristic acid. From both these acids γ-keto-acids were obtained and their identity has been confirmed by synthesis[1]. These results with the higher fatty acids suggested the extension of the enquiry to some of the lower members of the series, and the oxidation of hexoic, heptoic and octoic acids has been investigated. The separation of the products of oxidation yielded by these acids proved somewhat troublesome and so far is probably incomplete, but it has been satisfactorily established that each of them, besides undergoing β oxidation, as was shown by Dakin, also undergoes γ and δ oxidation. This has been proved by the isolation of the γ- and δ-keto-acids themselves, or their derivatives, from the products of oxidation and by their identity with specimens obtained by synthesis from substances of known structure. So far as can be determined from the results with these acids, β, γ and δ oxidations take place to about the same extent. Oxidation of fatty acids at the γ carbon atom has been previously observed with α-methylbutyric acid [Raper, 1914] and butyric acid [Cahen and Hurtley, 1917] when hydrogen peroxide was the oxidising agent. In both these instances it seemed likely that the terminal methyl group was oxidised because of its exposed position at the end of the carbon chain, especially as terminally oxidised fatty acids have been found in nature: *e.g.* hydroxypalmitic (juniperic) acid $CH_2OH(CH_2)_{14}COOH$ and hydroxylauric (sabinic) acid $CH_2OH(CH_2)_{10}COOH$ have been isolated from the waxes of certain coniferae [Bougault, 1910]; but the results described in the present paper suggest that oxidation of the γ carbon atom is a general feature when fatty acids are oxidised, and these two instances, therefore, are not exceptional. It is also of interest to note that Bödtker [1912] isolated γ-keto-sebacic acid in small amount when sebacic acid was oxidised with strong nitric acid.

Although hydroxy-acids are almost insoluble in light petroleum and should therefore be separable easily, it was never found possible to detect even traces of these acids; it was therefore concluded that the first step in the oxidation of a saturated acid is probably the production of a series of keto-acids and not hydroxy-acids.

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[1] For these syntheses we are greatly indebted to Professor and Mrs Robinson.

The further stages in the oxidation of γ- and δ-keto-fatty acids are at present under investigation; but one significant fact has already been discovered, namely, that both yield succinic acid as one of their oxidation products. This means that the carbon chain in these acids may break between the γ and δ carbon atoms. Four carbon atoms are thus split off as succinic acid.

These results are of interest from the biological point of view. They suggest that in the saturated acids, the β, γ and δ carbon atoms, and possibly also the α, are equally susceptible to oxidation, and that there is no marked difference in polarity between them as regards this property, though there certainly appears to be a marked one when ease of substitution by halogens is the criterion of polarity.

Whether all these forms of oxidation take place in the body we do not yet know. β oxidation certainly does, but there is practically no evidence in favour of α oxidation [Dakin, 1922]. If we accept the view that a substance placed in an environment with a suitably high oxidation potential is oxidised in a certain way which depends upon the structure of the substance and not upon the method by which the oxidation potential is obtained, then results obtained *in vitro* may be provisionally accepted as likely to take place in the body unless modified by some selective agency. That some such agency prohibits α oxidation seems very likely in view of the results of *in vivo* experiments, but the same cannot yet be said of γ and δ oxidation. If either of these processes occurred and were followed by rupture of the fatty acid chain between the γ and δ carbon atoms with the production of succinic acid, then four carbon atoms would be removed at a time instead of the usual two which are postulated by the β oxidation theory of breakdown. Such a process, associated also with β oxidation, would be quite compatible with the well-known results obtained by Embden and his co-workers [1906, 1908] with the surviving liver perfused with blood containing salts of the lower fatty acids. It would also be compatible with the results of Knoop [1904] and Dakin [1909] who showed that the oxidation of phenylvaleric acid in the body yielded benzoic acid. That a γ-keto-acid may lose four carbon atoms at a time on oxidation in the body is suggested very strongly by the results of Knoop and Oeser [1914], who determined the fate in the body of benzyllaevulinic acid. When 15 g. of this acid were administered to a dog, 3·6 g. of phenaceturic acid and 0·8 g. phenyl-α-hydroxybutyric acid were isolated from the urine. No hippuric acid was found. A study of the formula of benzyll-aevulinic acid

$$C_6H_5.\underset{\epsilon}{CH_2}.\underset{\delta}{CH_2}.\underset{\gamma}{CO}.\underset{\beta}{CH_2}.\underset{\alpha}{CH_2}.COOH,$$

shows that β oxidation would yield benzylpyruvic acid,

$$C_6H_5.CH_2.CH_2.CO.COOH,$$

which on reduction would give the phenyl-α-hydroxybutyric acid obtained in this experiment. That the fatty acid side chain did not split between the β and γ carbon atoms and so yield phenylpropionic acid is shown by the absence of benzoic (or hippuric) acid among the products recovered from the urine, since phenylpropionic acid yields benzoic acid when oxidised in the body. The main product isolated, namely, phenylacetic acid (excreted as phenaceturic acid) seems undoubtedly to have been produced by splitting of the chain between the γ and δ carbon atoms, the δ carbon being oxidised to carboxyl. Presumably the remaining four carbon atoms would be split off as succinic acid which is known to be readily oxidised in the body.

The production of succinic acid by the oxidation of fatty acids in the body would provide a means of explaining how fatty acids might give rise to carbohydrates. Succinic acid is known to be easily converted into fumaric and malic acids both in muscle and liver and the latter acid in phlorhizin diabetes is converted into glucose. Whether this takes place *via* lactic acid is uncertain, but if it does, then a process of oxidation of fatty acids can be visualised which, by yielding in turn succinic acid, malic acid and either lactic acid or glucose, would provide a scheme whereby fat could supply the energy for muscular exercise. There is no experimental evidence which shows that muscle can use fat directly and it appears that it must undergo some preliminary oxidative processes elsewhere in the body in order that it may be prepared for use in the muscle mechanism. The oxidative breakdown of fatty acids must therefore follow some scheme which provides substances which will be utilisable by muscle, *e.g.* lactic acid or carbohydrate. The scheme of β oxidation cannot be said to do this. It does not throw any light on the mechanism whereby fat can be transformed into carbohydrate, as it apparently must be if the current ideas on the working of the muscle mechanism are correct. The very common occurrence in the body of the enzyme succinoxydon, discovered by Batelli and Stern [1911], and its rapid action certainly seem to suggest that there is a greater use for it than the oxidation only of glutamic acid after this has undergone previous oxidative deamination to succinic acid.

Some of these problems and speculations which have necessarily arisen as a result of the experiments described in this paper are being investigated in the hope that they will throw light on some aspects of the metabolism of fatty acids which are at present obscure.

EXPERIMENTAL.

Oxidation of stearic acid. Two samples of pure acid (M.P. 69°, M.W. by titration in neutral alcohol 284, iodine number 0·1), obtained from Messrs Kahlbaum and Poulenc Frères, were oxidised and gave the same result. Stearic acid (10 g.), water (250 cc.), and ammonia (4 cc. 23 %) were heated under reflux in a 3 litre flask to 60° in a water-bath. Hydrogen peroxide (21 cc. 30 %) was then added and the temperature gradually raised to 90° (there is considerable frothing). Heating was continued for 4 hours. The liquid was acidified with phosphoric acid, cooled and the precipitated acids and ketone filtered off. The precipitate was converted into soaps with potash in 50 %

alcohol and the solution extracted five times with light petroleum, thus removing the β oxidation product, methylquindecyl ketone. The alcoholic solution of the soaps was then acidified with dilute sulphuric acid, diluted, cooled and filtered. The resulting acid was recrystallised once from a small quantity of methylated spirit when 6–7 g. of nearly pure stearic acid separated. The acid in the mother liquid was neutralised with soda, poured into water and the lead salts precipitated by adding 20 % lead acetate solution. The ether-soluble lead salts have not been fully dealt with. The lead salts insoluble in ether were converted into acids and the latter crystallised from light petroleum. The first fraction was shown to be a new acid which after three recrystallisations from light petroleum melted at 97°. This acid was also obtained directly by crystallisation of the mother liquor acids from light petroleum without the precipitation of the lead salts. A number of these oxidations were carried out and the high melting fraction accumulated until sufficient was obtained for complete analysis and identification, when it was shown to be γ-ketostearic acid. This acid was synthesised from stearolactone and melts at 97° and on mixture with the acid obtained by oxidation did not depress the melting point. Both acids gave an oxime, M.P. 84°, and the analysis and molecular weight of the acid produced by oxidation corresponded with the theoretical.

(Found $C = 72{\cdot}59, 72{\cdot}63$, $H = 11{\cdot}61, 11{\cdot}59$ %, $C_{18}H_{34}O_3$ requires $C = 72{\cdot}48$, $H = 11{\cdot}4$ %.)

A trace of another acid (possibly δ-ketostearic acid) was obtained, but not in sufficient quantity for identification. The amount of β and γ oxidation products actually separated and weighed represented about 6 % and 1 % respectively of the stearic acid used. The β figure is probably maximal whereas the γ figure is low, due to the difficulty of separation and to the fact that γ-ketostearic acid is readily further oxidised by peroxide. This latter oxidation is now being investigated. The constitution of γ-ketostearic acid has already been proved by the Beckmann reaction on its oxime [Shukov and Schestakov, 1903].

Oxidation of palmitic acid. Following the above method, an acid melting at 91–92° was isolated. This acid was more soluble in alcohol and less soluble in light petroleum than palmitic acid and corresponded in properties with γ-ketopalmitic acid which was kindly synthesised for us by Mrs Robinson [1925]. The mixed melting point was 91–92°.

Oxidation of myristic acid. The oxidation of this acid was carried out as above but owing to the lower melting point of the acid and the greater solubility in alcohol, the method of separation had to be modified. After separation of the ketone in the usual way, the fatty acids were crystallised from methyl alcohol, the liquid being cooled in ice whereby 60–70 % of unchanged myristic acid was recovered. The acid recovered from the mother liquor was crystallised from 80 % methyl alcohol and yielded a further amount of myristic acid. The acids remaining in the mother liquor were now crystallised from light petroleum and gave an acid, M.P. 87°, M.W. by titration 244. Calculated for $C_{14}H_{26}O_3$, 242. This acid has been synthesised by Mrs Robinson and from the M.P. and mixed M.P. has been proved to be γ-ketomyristic acid.

Oxidation of n-*hexoic* (*caproic*), n-*heptoic* (*oenanthic*) *and* n-*octoic* (*caprylic*) *acids.* The oxidations were carried out as before, the quantities employed being: acid (10 g.), water (250 cc.), ammonia (20 cc. 23 %), and hydrogen peroxide (50 cc. 30 %). As these acids are liquid at the ordinary temperature the fractional crystallisation method of separation had to be abandoned. In each case, the product of oxidation was extracted five times with light petroleum to remove the *n*-propyl-, *n*-butyl-, *n*-amyl-, methyl ketones formed by β oxidation. The liquid was acidified and again extracted five times with light petroleum. This removed the unchanged acid almost completely together with a trace of keto-acid. The resulting liquid was then extracted in a continuous ether extractor for 5–6 hours and the ethereal solution dried and fractionated. Some acetic acid was obtained in each case and also a main fraction boiling from 170–190° (20 mm.). This was shown to contain the γ- and δ-keto-acids. These were converted into the semicarbazones by adding to the oily fraction a little water, a considerable amount of crystalline sodium acetate and finally semicarbazide hydrochloride. The semicarbazones separated, were filtered off, dried and weighed. From the weight of ketones and of semicarbazones it was calculated that roughly 6 % of the acid had undergone β oxidation and 12 % $\gamma + \delta$ oxidation, and from the fractionation of the semicarbazones it appears probable that the amounts of β, γ and δ oxidation are roughly equal. The melting points (with decomposition) of semicarbazones are known to vary with the rate of heating, but by standardising the latter quite consistent results could be obtained [cf. Vorländer, 1897]. In the case of *hexoic* acid, the keto-acids obtained by oxidation gave a very strong iodoform reaction indicating the presence of δ-ketohexoic acid. The semicarbazones were separated by a long and tedious series of fractional crystallisations from methylated spirit into a least soluble portion, melting at 173°, shown to be δ-ketohexoic acid semicarbazone, a large number of intermediate fractions melting from 144–153° and a most soluble portion, melting at 170°, shown to be γ-ketohexoic acid semicarbazone. The following melting points and mixed melting points show very definitely that these acids were isolated as semicarbazones in practically pure condition:

Ketohexoic acid semicarbazone	M.P.
γ (synth.), γ (oxidn.)	170°
γ (synth.) + γ (oxidn.)	170
δ (synth.), δ (oxidn.)	173
δ (synth.) + δ (oxidn.)	173
δ (synth.) + γ (synth.)	145–148
δ (synth) + γ (oxidn.)	145–148
γ (synth.) + δ (oxidn.)	145–148
γ (oxidn.) + δ (oxidn.)	145–148

Dumas nitrogen estimations on the semicarbazones of the oxidation γ- and δ-ketohexoic acids thus separated, gave the following results:

(*a*) γ: found N = 22·3 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.

(*b*) δ: found N = 22·2 %; $C_7H_{13}O_3N_3$ requires N = 22·4 %.

The oximes of the synthetic γ-ketohexoic and δ-ketohexoic acids and of the oxidation mixture of these two acids were made by dissolving hydroxylamine hydrochloride (5 g.) in a small amount of water, adding the keto-acid (6 g.) and, after cooling, mixing with potash (7 g. in a little water) and allowing to stand overnight. The liquid was then acidified, saturated with ammonium sulphate and extracted in a constant ether extractor for 2 or 3 hours. The ethereal solution was dried over calcium chloride and the ether evaporated off. In the case of δ-ketohexoic acid, the oxime readily solidified and was recrystallised from benzene; M.P. 104°. In the case of γ-ketohexoic acid a syrup was obtained which, after standing 3 days over sulphuric acid *in vacuo*, on scratching with a glass rod gradually set to a solid. This was pressed on a porous plate and a little oily substance thus removed. The oxime was dissolved in dry ether, the solution filtered and then precipitated with light petroleum. After several purifications it melted at 67°. In the case of the keto-acids from the oxidation products the oxime was a thick syrup. This was dissolved in benzene and the oximes precipitated with light petroleum as an oil which, after inoculating with a very small crystal of δ-ketoxime, was allowed to stand 3 days *in vacuo* over sulphuric acid. Partial solidification took place and the mass was placed on a clean porous plate. The syrup was absorbed and a white solid residue obtained which crystallised readily from benzene, melted at 104°, and, mixed with synthetic δ-ketoxime, showed no depression of melting point. The absorbed syrup was recovered by extraction of the porous plate with benzene and the oxime precipitated with light petroleum and the process repeated. A little more δ-ketoxime was obtained and the syrup recovered after this operation was finally caused partially to solidify after inoculating with a small crystal of synthetic γ-ketoxime and allowing to stand *in vacuo* for several days. This fraction after purification was shown by mixed melting point to be the γ-ketoxime.

Analysis.

(*a*) γ: found N = 9·4 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

(*b*) δ: found N = 9·5 %; $C_6H_{11}O_3N$ requires N = 9·7 %.

In the case of *heptoic acid*, the semicarbazones of the oxidation keto-acids were separated as above and gave a less soluble first portion, M.P. 194°, identical with the synthetic δ-ketoheptoic acid semicarbazone and a much more soluble one, M.P. 152°, identical with γ-ketoheptoic semicarbazone. The mixed melting points of oxidation and synthetic semicarbazones were taken and the Dumas nitrogen analysis of the oxidation acid semicarbazones gave the following results:

(*a*) γ: found N = 20·8 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

(*b*) δ: found N = 21·1 %; $C_8H_{15}O_3N_3$ requires N = 20·9 %.

The ketoheptoic acids obtained by oxidation gave a slight iodoform reaction suggesting that a little ϵ oxidation had taken place. It was not found possible however to isolate with certainty a pure sample of the ϵ-semicarbazone (M.P. 144°) from the semicarbazone mixture.

In the case of *octoic acid*, similarly, the semicarbazone mass was by a tedious series of fractionations from alcohol separated into a less soluble first fraction of δ-ketooctoic acid semicarbazone, M.P. 187°, and a much more soluble fraction, γ-ketooctoic acid semicarbazone, M.P. 153°, neither of which depressed the M.P. of the corresponding synthetic semicarbazone. The following Dumas nitrogen determinations were carried out on the oxidation semicarbazones:

(*a*) γ: found N = 19·8 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

(*b*) δ: found N = 19·7 %; $C_9H_{17}O_3N_3$ requires N = 19·5 %.

Synthesis of keto-acids by means of zinc alkyl iodides.

The method adopted was similar to that of Blaise and Kohler [1910]:

$$RZnI + ClCO(CH_2)_nCOOEt = RCO(CH_2)_nCOOEt + ZnICl.$$

(i) *Preparation of the zinc alkyl iodides.*

Pure zinc filings (90 %) and pure copper filings (10 % of total weight) were heated together in a dry flask in the yellow blowpipe flame until the mass fell to a black powder. The alkyl iodide (1 mol.), ethyl acetate ($\frac{1}{3}$ mol., dried for several days over calcium chloride, distilled and first and last fractions neglected), dry redistilled toluene (double the weight of ethyl acetate) and Zn – Cu couple (double the amount required by the iodide) were heated in a dry flask under reflux at 100–110° for $1\frac{1}{2}$ hours. A second equal amount of toluene was added and, after shaking, the flask was cooled. The white fuming liquid was poured into a dry flask and the couple washed with a little dry toluene. In this way were prepared zinc ethyl-, *n*-propyl-, *n*-butyl-, iodides.

(ii) *Preparation of half ester half acid chlorides of*

(*a*) *Succinic acid.* Succinic anhydride (1 mol.) was boiled with methyl alcohol (2 mols.) for 45 minutes. The excess of alcohol was distilled off and the mass after solidifying was crystallised from a large quantity of carbon disulphide. The monomethyl ester (M.P. 58°) thus obtained was boiled with three times its weight of redistilled thionyl chloride, the excess of thionyl chloride distilled off *in vacuo* and the methyl ester chloride (B.P. 93°/18 mm.) was collected.

(*b*) *Glutaric acid.* Glutaric acid was prepared by a method, the details of which were kindly provided by Professor C. K. Ingold. Cyclohexanol

(500 g.) was added, drop by drop, with occasional shaking, to 2700 g. conc. nitric acid in a 5 l. flask provided with an efficient reflux condenser and heated in a boiling water-bath. After the reaction was completed (4–5 hours) the liquid was poured into a basin and allowed to stand overnight. The crystals of adipic acid were filtered off on a glass wool pad in a large Buchner funnel and thoroughly dried in the steam oven, powdered and heated in the oven for 48 hours completely to drive off the nitric acid. A little more was obtained from the mother liquors making the total yield of adipic acid 558 g. This experiment was repeated. Dry adipic acid (300 g.) and baryta (50 g.) were heated in a 1·5 l. distilling flask in an air-bath at 290°. The water coming over at first was neglected and the cyclopentanone collected as long as any liquid distilled (3–4 hours). This was shaken with ether, when a little water separated, and the ethereal solution was dried over calcium chloride and distilled, using a long fractionating column. The cyclopentanone collected at 129–133° weighed 118 g. This experiment was repeated. Cyclopentanone (200 g.) was dropped (as in the oxidation of cyclohexanol) into 2000 cc. of dilute nitric acid (sp.g. 1·2). The resulting liquid was evaporated to dryness, thoroughly dried and freed from nitric acid. The powder obtained was placed in a large Soxhlet thimble and suspended in a flask over boiling benzene, the condensed benzene dripping into the thimble. Almost pure glutaric acid, M.P. 95–96°, separated from the benzene solution on cooling. The extraction was continued until only succinic acid remained in the thimble. 1 kg. cyclohexanol gave 390 g. glutaric acid. Glutaric anhydride was prepared by heating dry glutaric acid with five times its weight of thionyl chloride, evaporating off the excess of the latter *in vacuo* and collecting the fraction boiling at 145–165°/20 mm. This fraction readily solidified and was crystallised from boiling ether when it melted at 55°. The anhydride was boiled with double the theoretical amount of methyl alcohol, the excess of alcohol was distilled off and the monomethyl ester collected at 153°/20 mm. This was boiled with three times its weight of redistilled thionyl chloride, excess of the latter was removed *in vacuo* and the methyl ester chloride collected at 110°/20 mm.

(iii) *Preparation of the keto-acids.*

The methyl ester acid chloride dissolved in its own volume of dry toluene was added drop by drop to the zinc alkyl iodide solution, the latter being about 25 % in excess for the acid chloride used. During the whole operation the reaction flask was kept in ice and shaken constantly. After standing, water was added, a little at a time at first, shaking vigorously. A little precipitated zinc hydroxide was dissolved in dilute sulphuric acid. The toluene solution was separated and washed with dilute ammonia, then with dilute sulphuric acid and finally with dilute bicarbonate and thiosulphate. The solution was dried over anhydrous sodium sulphate and fractionated *in vacuo*. The methyl esters of the keto-acids were then hydrolysed by shaking with strong potash, the product acidified and extracted with ether. (In the case of

γ-ketohexoic acid continuous ether extraction was necessary.) The ethereal solution was dried and fractionated *in vacuo*. In this way were prepared:

(*a*) *γ-ketohexoic acid*; using zinc ethyl iodide and the half-ester half-acid chloride of succinic acid. The acid was collected from 150–180°/20 mm. and set to a hard mass on standing *in vacuo*. This was dissolved in benzene, filtered from a trace of succinic acid and the benzene evaporated to a small volume, when the acid crystallised. Purification by precipitation from benzene solution by light petroleum was also tried. γ-Ketohexoic acid melts at 32°, boils at 180°/20 mm. and gives a semicarbazone melting with decomposition at 170°, which appears to be identical with that obtained by Campbell and Thorpe [1910]. The oxime melts at 68°. The methyl ester boils at 110°/20 mm.

Analysis.

Semicarbazone: found $N = 22{\cdot}35\ \%$; $C_7H_{13}O_3N_3$ requires $N = 22{\cdot}4\ \%$.
Oxime: found $N = 9{\cdot}9\ \%$; $C_6H_{11}O_3N$ requires $N = 9{\cdot}7\ \%$.

(*b*) *γ-ketoheptoic acid*; using zinc propyl iodide and the half-ester half-acid chloride of succinic acid. The acid melts at 48° and gives a semicarbazone, M.P. (decomp.) 152°. The methyl ester boils at 125°/20 mm.

Analysis.

Semicarbazone: found $N = 21{\cdot}1\ \%$; $C_8H_{15}O_3N_3$ requires $N = 20{\cdot}9\ \%$.

(*c*) *γ-ketooctoic acid*; using zinc butyl iodide and the half-ester half-acid chloride of succinic acid. The acid melts at 53° and gives a semicarbazone, M.P. (decomp.) 153°. The methyl ester boils at 111°/15 mm.

Analysis.

Semicarbazone: found $N = 19{\cdot}8\ \%$; $C_9H_{17}O_3N_3$ requires $N = 19{\cdot}5\ \%$.

(*d*) *δ-ketoheptoic acid*; using zinc ethyl iodide and the half-ester half-acid chloride of glutaric acid. The acid melts at 50° and gives an oxime M.P. 118° and a semicarbazone, M.P. (decomp.) 194°. The methyl ester boils at 130°/20 mm.

Analysis.

Semicarbazone: found $N = 21{\cdot}2\ \%$; $C_8H_{15}O_3N_3$ requires $N = 20{\cdot}9\ \%$.

(*e*) *δ-ketooctoic acid*; using zinc propyl iodide and the half-ester half-acid chloride of glutaric acid. The acid gives a semicarbazone, M.P. (decomp.) 187°. The methyl ester boils at 120°/15 mm.

Analysis.

Semicarbazone: found $N = 19{\cdot}7\ \%$; $C_9H_{17}O_3N_3$ requires $N = 19{\cdot}5\ \%$.

Synthesis of δ-ketohexoic acid.

The method adopted was essentially that of Bentley and Perkin [1896]. Sodium (3·4 g.) was dissolved in alcohol (45 g.) and to the well-cooled solution acetoacetic ester (19 g.) and then β-iodopropionic ester (33 g.) were added. After standing for half an hour the whole was heated on a water-bath for

2 hours, water added and the oil extracted with ether. The ethereal solution was washed with a little water containing SO_2, dried over $CaCl_2$, evaporated and the oil fractionated *in vacuo*. Acetylglutaric ester was collected at 190–200°/50 mm. and boiled under reflux with five times its volume of dilute (1 : 2) HCl for 5 hours. The liquid was saturated with ammonium sulphate and extracted six times with ether. The ethereal solution was dried over $CaCl_2$ and fractionated, the keto-acid being collected at 195–200°/65 mm. The acid is an oil which forms a crystalline monohydrate, M.P. 35°, and very readily gives the iodoform reaction. The semicarbazone melts at 173° and the oxime at 103°.

Analysis.

Semicarbazone: found $N = 22{\cdot}6\ \%$; $C_7H_{13}O_3N_3$ requires $N = 22{\cdot}4\ \%$.
Oxime: found $N = 9{\cdot}6\ \%$; $C_6H_{11}O_3N$ requires $N = 9{\cdot}7\ \%$.

Synthesis of γ-ketostearic acid. Stearolactone, M.P. 52°, was prepared by the action of sulphuric acid on oleic acid [Schukov and Schestakov, 1908] and oxidised to γ-ketostearic acid by means of chromic acid in acetic acid solution [Clutterbuck, 1924].

The acid melts at 97° and was converted into its oxime as follows: γ-ketostearic acid (1 mol.), hydroxylamine hydrochloride (1 mol.) and caustic soda (3 mols.) in alcoholic solution were boiled for 2 hours on the water-bath. The alcohol was distilled off, the residue dissolved in water and, after cooling, made just acid. The precipitated acids, after extraction with ether and removal of the latter, were boiled with light petroleum in which the oxime was more soluble than the unchanged keto-acid. The oxime when pure melted at 85°. The reaction was studied under varying conditions but the yield was never theoretical and consequently oxime formation could not be used for the quantitative estimation of γ-ketostearic acid in the oxidation mixture.

Analysis. Found $N = 4{\cdot}29\ \%$; $C_{18}H_{35}O_3N$ requires $N = 4{\cdot}49\ \%$.

γ-Ketostearic acid phenylhydrazone was prepared by dissolving the keto-acid in warm glacial acetic acid and adding a few drops of phenylhydrazine. The hydrazone crystallised out on standing and was recrystallised from light petroleum. It melts with total decomposition at 80° and therefore great care was required in its purification.

Analysis. Found $N = 7{\cdot}0\ \%$; $C_{24}H_{40}O_2N_2$ requires $N = 7{\cdot}2\ \%$.

Synthesis of γ-ketopalmitic and γ-ketomyristic acids. These were kindly prepared by Mrs Robinson [1925]. The former melts at 91–92° and gives an oxime, M.P. 54°. The latter melts at 87°.

SUMMARY.

(1) The ammonium salts of normal saturated fatty acids when oxidised with hydrogen peroxide yield products which show that oxidation occurs at the γ and δ carbon atoms as well as the β carbon atom (and possibly also the α).

(2) From myristic, palmitic and stearic acids, the corresponding γ-keto-acids have been isolated as products of oxidation.

(3) From *n*-hexoic, *n*-heptoic and *n*-octoic acids the corresponding γ- and δ-keto-acids have been isolated as semicarbazones and in some cases also as oximes. Several of these acids had not been previously prepared and have been synthesised for reference.

(4) The possibility of the occurrence of γ and δ oxidation in the body is discussed and it is pointed out that if further investigation proves that it does occur, some light may be thrown on the processes by which fat may be utilised to supply energy for the contraction process of muscle.

(5) The first step in the oxidation of normal saturated fatty acids appears to be the formation of a series of keto-acids and not of hydroxy-acids. The latter, although almost insoluble in light petroleum, could not be detected. This would support the view that the hydroxy-acids obtained in experiments *in vivo* result from reduction of keto-acids rather than from direct oxidation of saturated acids.

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LVIII. STUDIES ON THE PHYSIOLOGY OF PLAIN MUSCLE.

III. COMPARISON OF THE REDUCING PROPERTIES OF PLAIN AND STRIATED MUSCLE[1].

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The purpose of the present experiments was to study the reducing properties of plain and striated muscle in order to ascertain whether there were any pronounced differences in this respect which would be of value in the consideration of their comparative physiology. In making the comparison, the samples of plain and striated muscle have in each case been taken from the same animals, sometimes the frog, more often a mammal, and have been subjected to identical treatment. The actual striated muscle chosen for comparison has varied; in cats the muscle of the thigh, in frogs the sartorius or gastrocnemius, in the bullock, portions of perineal muscle, etc. In view of the greater variability of plain muscle, as regards its physiology, various sources have been used, but no conspicuous differences between them were seen. The tissues used were small intestine, uterus, stomach, and the retractor penis. The reductions studied were those of methylene blue, and of *m*-dinitrobenzene, the former by the well-known technique introduced by Thunberg [1920], the latter as carried out by Lipschitz [1920]. When possible the experiments were carried out on fresh tissues; when material had to be kept it was immersed in 2 % boric acid and kept in the refrigerator; previous to use such preserved muscle was well washed with several changes of distilled water in a shaking machine; such material served very well for the study of the reductions of washed tissue.

It may be said at the outset that the qualitative differences which were found between the two varieties of muscle were negligible, the only important differences being quantitative.

THE REDUCTION OF METHYLENE BLUE.

The reduction of methylene blue *in vacuo* at 35° is usually effected by plain muscle in the fresh state with a velocity only about one-third to one-fifth of that brought about by striated muscle under identical conditions. The following table shows the result of a typical experiment:

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[1] Protocols of experiments have been abbreviated or omitted in order to save space, as the technique employed is fully described in the papers quoted.

Table I. *Reduction of Methylene Blue by Fresh Muscle.* (*Cat. Intestine and thigh muscle.*)

Mg. m. bl.	0·02	0·02	0·04	0·04	0·06	0·06	0·08	0·08
Plain mus. (g.)	0·2	—	0·2	—	0·2	—	0·2	—
Str. mus. (g.)	—	0·2	—	0·2	—	0·2	—	0·2
Time for reduction (mins.)	15	4	11	12	40	—	40	17

Mg. m. bl.	0·04	0·04	0·04	0·04	0·04	0·04	0·04	0·04
Plain m. (g.)	0·1	—	0·2	—	0·3	—	0·4	—
Str. mus. (g.)	—	0·1	—	0·2	—	0·3	—	0·4
Time for reduction (mins.)	>43	21	28	5	27	5	12	1

It will be seen that the plain muscle is much inferior in its reducing power to striated muscle, both when compared with varying amounts of the dye, and also when the amount of tissue is changed, the concentration of dye being kept constant. This result is not surprising in view of the relatively small oxygen usages of plain muscle reported by Evans [1923]. Similar results were obtained with frog tissues; thus in one experiment, using 0·2 g. tissue and 0·4 mg. methylene blue, the times for reduction were 40 minutes for plain muscle, 7·5 minutes for heart muscle, and 5·5–8·0 minutes for striated muscle.

When the finely minced plain muscle is well washed with several changes of water, its reducing power is almost completely lost; this happens also with the striated muscle. The reduction can, however, be restored again to a considerable extent by addition of potassium succinate as also holds good for striated muscle. One sample of plain muscle (0·2 g.) for instance in the fresh state reduced 0·04 mg. methylene blue in 28 minutes; after washing well with water the same tissue (0·2 g.) showed no sign of reduction of 0·02 mg. methylene blue after 1 hour; when 0·2 cc. of 0·1 molar potassium succinate was added, the reduction under otherwise similar conditions was almost complete in 50 minutes.

Optimal Reaction.

When buffered with phosphate solutions, the optimal rate of reduction was obtained with striated muscle at a p_{H} above 10·5, whilst with plain muscle the optimum was about p_{H} 9·0–10·0. With borate buffers the optimal p_{H} was about 8·0 for plain, and about 8·8 for striated muscle. In both instances washed tissue in presence of succinate was used for these tests. The following is the result of the experiments: in each case 0·2 g. of the washed tissue, 0·1 cc. 1 : 5000 methylene blue, 0·1 cc. of $M/10$ potassium succinate, and 0·9 cc. of $M/10$ potassium phosphate buffers were taken. The reduction times were:

p_{H}	For striated muscle	For plain muscle
3·0–6·7	>60′	>72′
7·2	27′	61′
7·7	28′	37′
9·0	24′	26′
10·5	15′	36′

Alteration in the amount of buffer solution made no appreciable difference to the result, *e.g.* the reduction times were the same with 1 cc. of buffer solution as with 0·2 cc. buffer solution and 0·8 cc. water.

Effect of Temperature.

The optimal temperature, at p_H 9·0 is, according to my experiments, about 50° for both varieties of muscle. Thus in a mixture containing 0·2 g. washed muscle, 0·1 cc. of 1 : 5000 methylene blue, 0·8 cc. of $M/10$ phosphate buffer, and 0·1 cc. of $M/10$ potassium succinate, the reduction times (brought to the same scale for purposes of comparison where different samples were used) were as follows: at 0°, the reduction was scarcely apparent, even after 18 hours; at 20° it was partially complete in 4 hours, at 30° complete in 4 hours, at 40° in 50 minutes, at 50° in 23 minutes, and at 60° in 32 minutes. Experiments were, however, usually carried out at 38°.

Effect of Salts and of KCN.

The reduction of methylene blue by washed plain muscle, in presence of succinate, is accelerated by both sodium and potassium chlorides, precisely as with striated muscle. The effect of cyanide is also identical, that is to say, the reduction by the washed tissue (0·2 g.) of methylene blue (0·2 cc. of 1 : 5000) in presence of succinate (0·2 cc. of $M/10$ potassium succinate) is not inhibited, but slightly, though definitely, accelerated by the presence of potassium cyanide in concentrations up to 0·2 cc. of $M/100$ KCN in 1 cc. of reaction mixture. The reduction was carried out in presence of 0·4 cc. of $M/10$ K_2HPO_4, and the times for complete reduction were, in one experiment, without cyanide 2 hours; with 0·1 cc. $M/1000$ cyanide, 1 hour 24 minutes; with 0·2 cc. $M/1000$, 1 hour 13 minutes, with 0·05 cc. $M/100$, 1 hour 19 minutes; with 0·1 cc. 1 hour 11 minutes, and with 0·2 cc., 1 hour 15 minutes. Other experiments gave similar results, there being in all cases definite acceleration of the reduction after addition of KCN. The acceleration was possibly due to slight reduction of the H-ion concentration of the mixture, but was not further investigated, the chief point being that there was no inhibition of the reduction. In this respect also there is a close resemblance to the behaviour of striated muscle, as studied by Thunberg [1917].

Restoration of Reducing Power by Addition of Organic Acids.

When plain muscle is washed in the manner described by Thunberg [1920], its reduction power is almost completely lost. On addition of potassium succinate the reducing power is partially restored; but no matter how much succinate is added to it, the reducing power does not approximate to that of the fresh tissue, and still less to that of fresh striated muscle.

It appeared of interest, in connection with the results obtained by Thunberg, to find whether the substances which were capable of restoring the power of reduction to plain muscle inactivated by washing were the same as those which acted in that way with washed striated muscle. In addition to succinate, I tried lactate, citrate, acetate and formate. The experiments were carried out in every respect according to the Thunberg technique, and need not be described in any detail; in each instance a parallel experiment was carried out with washed striated muscle to serve as a control. The amounts of materials placed in the evacuated tubes were as follows: muscle 0·2 g. $M/10$ potassium lactate (or other organic salt) solution and water together to make up 0·4 cc.; $M/10$ K_2HPO_4 solution 0·5 cc.; methylene blue (1 : 5000) 0·1 cc. Temp. 37°.

The results, at the reaction given by this mixture (about p_{H} 9) may be summarised as follows:

Plain muscle		Striated muscle	
Reduction time		Reduction time	
No lactate	Lactate (0·4 cc.)	No lactate	Lactate (0·2 cc.)
+ +in 4 h. 19 m.	Complete in 4 h. 9 m.	+ +in 4 h. 7 m.	Complete in 4 h. 2 m.
No formate	Formate (0·1–0·4 cc.)	No formate	Formate (0·1–0·4 cc.)
0 in 2 h.	0 in 2 h.	0 in 2 h.	0 in 2 h.
No acetate	Acetate (0·1–0·4 cc.)	No acetate	Acetate (0·1–0·4 cc.)
+in 3 h.	+in 3 h.	+in 3 h.	+in 3 h.
No citrate	Citrate (0·4 cc.)	No citrate	Citrate (0·5 cc.)
+ +in 3 h. 27 m.	+in 3 h. 23 m.	+ + +in 3 h. 22 m.	+ + +in 3 h. 16 m.

These results show that there was a slight acceleration of reduction by lactate, but, at this hydrogen ion concentration no effect produced by acetate, formate or citrate. The effect of altering the H-ion concentration was, however, quite considerable with citrate, which, as shown by Fleisch [1924], is oxidised by washed muscle and methylene blue much more rapidly at a p_{H} between 5·0 and 6·5 than at 7·6. I have similarly found that whilst at p_{H} 9·0 and 7·5 citrate was not oxidised, there was pronounced decolorisation of the methylene blue at p_{H} 6·3 and 5·5, *e.g.* in experiments with quantities as above, and using washed plain muscle:

At p_{H} 5·5 without citrate; only slight decolorisation in 3 hours 38 minutes.

At p_{H} 5·5 with 0·1 cc. citrate; decolorisation almost complete in $3\frac{1}{2}$ hours.

At p_{H} 6·3 without citrate; slight decolorisation in 3 hours 37 minutes.

At p_{H} 6·3 with 0·3 cc. citrate; almost completely decolorised in $3\frac{1}{2}$ hours.

At p_{H} 7·5 without citrate, or with 0·6 cc. citrate; decolorisation nearly complete in $3\frac{1}{2}$ hours.

With lactates it was found that the acceleration of decolorisation was most rapid at p_{H} 8·0–9·2.

There does not seem to be any noteworthy difference between the properties of striated and those of plain muscle in these respects. I could not confirm the oxidation of acetates and formates by either tissue, though a definite action by striated muscle was found by Thunberg [1920].

REDUCTION OF *m*-DINITROBENZENE.

A comparison was also made between the reducing power of plain and striated muscles and of other tissues, on *m*-dinitrobenzene. W. Lipschitz [1920, 1921] and his collaborators have shown that this substance is reduced by many tissues to the soluble, yellow *m*-nitrophenylhydroxylamine, the amount of which may be estimated colorimetrically. This reduction, unlike that of methylene blue, is not reversible by atmospheric oxygen, though it takes place more readily in the absence of oxygen, and in most ways gives results which are similar to those given by methylene blue reduction.

It was found, using a method similar to that of Lipschitz, that plain muscle reduced the *m*-dinitrobenzene with much greater speed than did striated muscle. Comparison of various tissues showed that with cat's tissues the amounts of reduction obtained in equal lengths of time were in descending order: plain muscle of small intestine; heart muscle; uterus muscle; striated muscle. With frog's tissues liver and testis reduced more rapidly than muscular tissue, plain and striated muscle showing little difference in this experiment. In all the experiments with mammalian tissues however it was found that the plain muscle showed upwards of twice as much reduction in a given time as was shown by striated muscle. In all these experiments the amounts of substances used were, muscle 0·5 g., total fluids 3·0 cc. and *m*-dinitrobenzene, in finely powdered form, 0·05 g. It was found that the reduction with striated muscle soon reached a standstill, whereas that given by the plain muscle was able to proceed for a longer time, as well as at a more rapid rate; it seems highly probable that this remarkable difference between the two tissues, which behave in the opposite way towards methylene blue reduction, may be due to the fact that the reduction product is for some reason more toxic to striated muscle than to plain muscle, and so brings the reduction reaction to an end as soon as it has accumulated in sufficient amount. It was found, in agreement with Lipschitz, that the reduction took place in all instances much more rapidly *in vacuo* than when the contents of the tube were exposed freely to the air. On thorough washing with water, the reducing power was completely lost, but was partially restored by addition of succinates, just as with methylene blue reduction. The reduction was not inhibited by reasonably small amounts of cyanide, such as $M/400$ KCN, or less.

This fact is not in agreement with the observations of Lipschitz and Gottschalk [1921] who found considerable inhibition at low concentrations of cyanide with frog's striated muscle.

THE REDUCING POWER OF AQUEOUS EXTRACTS.

Aqueous extracts of fresh plain and striated muscle were made by treating the minced tissue with twice its weight of water, to which some boric acid was added when it was to be kept at room temperature for many hours, or on ice for many days. The extraction was carried out at 0° as a rule, but in some instances at room temperature or at 60–70°. The filtered extracts obtained differed in appearance, that from plain muscle being colourless but opalescent, while that from striated muscle was red, owing to the presence of haemoglobin, and clear. In some of the experiments filtration was carried out through a Berkfeld filter, but the results were the same as when the filtration was through paper alone. The filtered extracts showed definite reducing power towards methylene blue *in vacuo*, but the plain muscle extract had much the more powerful effect in this respect. Thus in one experiment 0·2 g. of fresh plain muscle reduced 0·02 mg. of methylene blue in 1 cc. water in 35 minutes; the same amount of striated muscle brought about the reduction in 8 minutes. An extract of samples of each tissue was made at 60° with twice its weight of water, and filtered after 1 hour. To 0·9 cc. of each filtrate 0·1 cc. of methylene blue (0·02 mg.) was added; reduction occurred *in vacuo* in 21 minutes with the plain muscle extract, whilst the striated muscle extract showed no sign of reduction after 36 minutes. On adding to 0·9 cc. of each extract 0·2 g. of washed muscle from which the extracts had been made, and 0·1 cc. of the methylene blue solution, there was reduction in 15 minutes with the plain muscle extract plus washed plain muscle, and in 20 minutes incomplete reduction with the striated muscle extract to which the washed striated tissue had been added. It was found that plain muscle extract re-activated washed striated muscle much more than striated muscle extract. Parallel with this greater reducing and re-activating power of the plain muscle extract is a much more intense nitroprusside reaction for glutathione [Hopkins, 1921], and the fresh tissues themselves also show the same difference in this respect when tested directly. On boiling, or on being allowed to stand in air for 2 or 3 days the extract loses its reducing power, and no longer shows the nitroprusside reaction; it is however still able to some extent to restore the reducing power to washed tissue. When the boiled extract is treated with a little fresh tissue ($\frac{1}{10}$th its weight), and again filtered off after half an hour, it is found that the reducing power and the nitroprusside reaction have been restored to the extract. It is evident from a consideration of these results that whilst the fresh extract of plain muscle contains a relatively large amount of gluthathione, this is lost by oxidation on standing, but can be converted to the reduced form again by treatment with either fresh or washed tissue [Hopkins, 1921]. It was also found that the reduction of washed tissue was accelerated to a greater extent when plain muscle extract and potassium succinate were added than when either of these was added alone; in fact in one experiment the reduction of methylene blue by washed muscle and fresh extract was no quicker than that by the extract alone.

Apparently some other factor is concerned in addition to glutathione, because it was found that when the extract was heated to 65° for 30 minutes in slightly acid solution, and then neutralised, much of its reducing power had been lost, although the nitroprusside reaction remained as intense as before. This was possibly due to destruction of some soluble catalyser or hydrogen acceptor.

CONCLUSIONS.

1. Plain muscle reduces methylene blue more slowly, but *m*-dinitrobenzene more quickly than striated muscle. Neither reduction is inhibited by cyanide.
2. Both types of reduction are almost abolished by washing, but are partly restored by addition of succinate.
3. The optimal p_H for plain muscle reduction of methylene blue is about 9·0.
4. The differences between the reducing action of plain and striated muscle appear to be quantitative rather than qualitative. It seems unlikely that there is any essential difference between them as regards the nature of their intermediary metabolic products.
5. Plain muscle and its extracts are richer in glutathione than striated muscle or its extracts, and show properties in accordance with this.

I hope to continue these experiments in the near future on my return to Japan.

My thanks are due to Professor C. Lovatt Evans for allowing me the facilities of his Department during this investigation.

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LIX. ANIMAL QUINOIDINE.

PRELIMINARY PAPER.

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It is a well-known fact that the nails, skin, teeth and bones fluoresce blue when exposed to a source of ultra-violet radiation. This is best seen by interposing between the source of radiation and the irradiated object a sheet of material which excludes the visible rays. While examining the properties of some of the yeast concentrates obtained in the course of another research, it was observed that these also showed a blue fluorescence. Specimens of normal urine are also known to behave similarly. The coincidence of these facts led to this work. We have found the following literature upon the subject of blue fluorescence in ultra-violet light in the animal organism.

HISTORICAL.

Brucke [1845] stated that the lens of the human eye absorbed blue rays. Helmholtz [1855] found the human retina to be feebly fluorescent. Regnauld [1858] using sunlight described a blue fluorescence in the ultra-violet in the lens of the sheep, dog, cat and rabbit, the central part of the lens retaining this property upon keeping. This did not hold for aquatic vertebrates or mollusca. In the case of the human eye, Setchenow [1859] found that the lens fluoresced strongly like quinine, the blue tone predominating. The cornea on the contrary was only feebly fluorescent and the aqueous humour did not fluoresce at all. The most interesting paper is one by Bence Jones [1866]. In a lecture to the Royal Institution, he described the presence of a substance in animal tissues to which he gave the name of "animal quinoidine." In the course of a research upon the rate at which quinine was distributed to the tissues after administration, he found that all animal tissues and organs contained a substance which fluoresced blue when excited by ultra-violet rays. Its behaviour resembled that of quinine in that the fluorescence was diminished by addition of salt or strong acid, being resistant to treatment with permanganate in acid solution. By comparison with the fluorescence of known concentrations of quinine salts he estimated the amount of this substance in various tissues.

Other work upon the nature of blue fluorescent substances seems to be scanty. Schliess and Lowenfeld [1863] described the blue fluorescence of urine in sunlight. Jaffé [1869] mentioned the blue fluorescence of urine, but the paper in question deals mainly with the properties of urobilin and the green fluorescence of bile salts. In modern times Schanz [1921] investigated the fluorescence of the lens and Langecker [1922] stated that most urines show a blue fluorescence and some a yellow. Familiar urinary constituents such as urochrome, uroerythrin, urobilin, and the porphyrins were not considered to be responsible for the blue fluorescence. In the case of the porphyrins, he prepared a series of salts of haematoporphyrin by the Milroy technique most of which gave a yellow or red fluorescence, the only exceptions to this being the copper salt which fluoresced violet in amyl alcohol, and the lead salt which fluoresced green.

EXPERIMENTAL.

The present paper gives an account of certain observations which we have made upon the nature and distribution of substances which fluoresce blue in ultra-violet light (Å. 3000–4000). Our observations are qualitative only so far as this paper is concerned, and may be grouped for convenience under the following headings:

A. Substances of known constitution, and certain commercial preparations.
B. Animal products: (1) (*a*) Guano, (*b*) Urine, (*c*) Bile.
    (2) Other animal extracts.
    (3) (*a*) Gelatin, (*b*) Skin, (*c*) Lens.
C. Plant products: (1) Grass.
    (2) Oils.
    (3) Other products.

Throughout we have employed as a source of ultra-violet radiation a mercury vapour quartz lamp made by the Hewittic Company, and have used as a screen for the exclusion of the visible rays a plate of glass supplied by Messrs Chance.

According to the assertion of the makers, this screen lets through light of wave length Å. 3100–3900, the maximum being at a wave length of 3650, at which there is a transmission of some 50 %. Solutions have been examined for fluorescence in thin walled test tubes 13 × 75 mm., having a thickness of glass of some 0·5 mm. Controls in quartz tubes have not shown different behaviour as regards the colour of the fluorescence.

A. *Substances of known constitution, and certain commercial preparations.*

Table I (*a*) is a list of substances of known constitution which have shown no fluorescence in solution. In the case of the amino acids care was taken to examine the behaviour in alkaline and acid as well as in neutral solution. Alcohol was added to obtain solution where necessary.

Table I (*b*) shows certain other substances found to fluoresce a faint blue.

Table I (*a*). *Substances showing no blue fluorescence in solution.*

Tryptophan, cystine, leucine, histidine, tyrosine, glycine.

Allantoin, caffeine, hypoxanthine, uric acid, creatine, creatinine, xanthine, alloxan, parabanic acid, guanine, urea, guanidine, hippuric acid.

Cholesterol, cholesterol acetate, cholic acid, cholalic acid, laevulose, arabinose, cane sugar (purified by alcohol treatment), mannitol, sodium propionate, sodium maleate, salol, salicin, amygdalin, calcium lactate, starch, crotonic acid, tripalmitin, sodium glycerophosphate, turpentine, histamine, triphenylguanidine, choline, ox blood plasma and serum[1], egg albumin.

Table I (*b*). *Substances showing blue fluorescence.*

Guanine (commercial specimen; due to impurity), yeast nucleic acid (comm. specimen), lecithin (comm. specimen; very faint), crude lanolin, sodium glycocholate and taurocholate (laboratory specimens, faint blue): in the case of the glycocholate, it was found that the fluorescence could be removed by shaking with chloroform.

Gelatin, Nelson's "gold label" (commercial).

Gelatin from ossein
Gelatin from Coignet "gold label" } flocculated in an electric field.
Gelatin from horn

(In the flocculated gelatin, the fluorescence was much less decided than in the commercial gelatin.)

B. *Animal products.*

1. (*a*) *Guano.*

We were led to investigate guano extracts from the observation that a commercial specimen of guanine fluoresced a blue colour. Fluorescent extracts can be obtained from guano in several ways of which the following two are typical examples:

Method 1. Guano is extracted with warm 50 % alcohol containing 1 % HCl. From the cooled and filtered extract, the addition of either ether or chloroform extracts the bulk of the blue fluorescent substance, some however always remains behind in the aqueous phase.

Method 2. Guano is extracted with warm acetone. From the cooled and filtered extract practically all the blue fluorescent matter passes into the ethereal layer, upon addition of ether and water, which, upon evaporation, yields a reddish brown residue fluorescing almost a quinine blue when diluted with alcohol containing water to a faint yellow colour. It is relatively insoluble in water in acid solution, but dissolves in quantity upon the addition of alkali. It has not been possible so far to separate the blue fluorescing substance from the pigmented matter. Weight for weight the crude fraction is at least five times more powerful than quinine sulphate as a source of blue fluorescence. Porphyrin is present in this fraction, and can be extracted from it by the method described by Fischer [1923] for the extraction of Kammerer's porphyrin, after solution in ether containing acetic acid. After removal of the porphyrin there are no bands seen in the extracts of fluorescent material, merely a vague absorption up to λ 490.

By treatment of the aqueous phase fractions obtained in method 1 with alcoholic potash, and subsequent shaking with ether, an ethereal extract is obtained which fluoresces green, changing to blue on neutralisation.

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[1] Dr Howitt mentioned to us that he has found blue fluorescence in human serum. We therefore examined rabbit serum and found fluorescence. The matter is being further investigated.

(*b*) *Urine.*

All the urines examined fluoresced a decided blue though this tint is slightly modified by the green fluorescence of urobilin. The urochrome fraction prepared by the Garrod technique [1894] fluoresces blue when diluted to a pale yellow.

As in guano, there are present two classes of fluorescent fractions, distinguished by their behaviour on fractionation from a water-alcohol-ether mixture. There is relatively more of the aqueous fraction present in the urines examined by us than in the guano extracts. The behaviour of the aqueous fraction on saponification with alcoholic potash is similar to that of the guano fraction.

(*c*) *Bile.*

Bile was treated with acetone and the precipitate removed. When this was shaken with ether in successive portions a deep violet blue fluorescence appeared in the ether phase quite different from the blue fluorescence previously mentioned. We obtained some evidence that there was also a "blue fluorescent substance" present in the aqueous layer, but it is difficult to see, as it is much obscured by bile pigment. It will be noted from Table I that specimens of cholalic and cholic acids showed no fluorescence. Laboratory specimens of Na taurocholate and glycocholate gave a slight blue fluorescence, quantitatively of a different order from the guano extracts. This appeared to be due to an impurity, as, in the glycocholate, the blue fluorescence passed readily into a chloroform layer upon shaking the aqueous solution with chloroform.

2. *Other animal extracts.*

We have obtained chloroform extracts fluorescing blue from sheep's liver, milk, and white hen feathers by extraction with acid alcohol followed by treatment with chloroform, and also from caseinogen by extraction with 95 % alcohol. Blood plasma and serum when diluted showed no fluorescence.

The extracts obtained under (1) and (2) are very similar in type. We have obtained evidence of a different behaviour in the case of (3).

3. (*a*) *Gelatin.*

Commercial leaf gelatin in the solid state and when dissolved in water fluoresces blue. The fluorescence of the solid gelatin is intensified by soaking in acetone. The blue fluorescence is hardly affected by boiling with dilute acids, but disappears in large part after treatment with alcoholic potash. We have not been able to obtain by any means an ether-alcohol extract from gelatin, which fluoresces blue. Further samples of flocculated gelatin kindly supplied by Prof. Schryver fluoresced a faint blue, but much less intensely than the commercial gelatin.

(*b*) *Skin.*

The skin used came from the calf and was freed from subcutaneous tissue as far as possible. Pieces of skin some 12 inches square were used for the experiments. The skin fluoresced an intense quinine blue when examined in the ultra-violet light.

Exp. 1. Skin was heated with 50 % alcohol containing 1 % HCl. None of the fluorescing material was extracted. After standing for some days the alcoholic extract was removed and the skin treated with alcoholic potash for 15 minutes. The mixture was neutralised with acetic acid and HCl. By extraction of the neutral extract with ether, blue fluorescence was seen in the ether layer, but the greater part remained in the aqueous phase.

Exp. 2. Skin was hydrolysed with alcoholic potash as a first step. The hydrolysate was evaporated nearly to dryness on the water bath. An aqueous solution of the residue fluoresced blue. When the alkaline solution was shaken with ether, there was seen a yellowish blue fluorescence in the ether layer. After neutralisation of the solution with acetic acid, the ethereal layer fluoresced blue.

Exps. 1 and 2 indicate that though there is present in skin some blue fluorescent substance which will pass into ether, there is also a large amount of material which will not pass into an ether layer. Exp. 3 shows that this substance can be extracted with dilute acid.

Exp. 3. Skin was heated with dilute aqueous HCl in an attempt to follow the technique of Bence Jones. In this way an extract of a gelatin type was obtained which fluoresced an intense blue, and was readily thrown out of solution by alcohol. The acid extract was concentrated and treated with acetone to dry it. It was then extracted under the reflux condenser with boiling absolute alcohol and ether successively. Neither of these extracts fluoresced. After removal of the ether, the fluorescent residue was hydrolysed with 5 % HCl for some hours. After this the fluorescence was still present though it seemed to be diminished in intensity. Upon neutralisation, again no fluorescent material could be extracted by ether. The acid hydrolysate was neutralised and treated with alcoholic potash, after which treatment again no fluorescent ethereal extract could be obtained.

From these experiments we conclude that though there are present traces of substances in the skin which will yield "blue fluorescent" ether extracts, the main part of the blue fluorescence of skin is due to some other constituent in which fluorescence is associated with a gelatin-like substance.

(*c*) *Lens.*

The substance causing blue fluorescence of the lens of the sheep and ox could not be extracted with acid 50 % alcohol, and behaved similarly to the acid aqueous extract of skin in that it could not be extracted by ether, and was largely destroyed upon saponification with alcoholic potash.

C. *Plant Products.*

(1) *Grass.*

Grass leaves were treated as described by Plimmer according to the Willstätter technique for the preparation of chlorophyll, xanthophyll and carotene. None of these extracts fluoresced blue; chlorophyll was purplish red, xanthophyll yellow and carotene greenish yellow when examined under our experimental conditions. An extract of carotene prepared from carrots behaved similarly. We found, however, that the first acetone extract of the leaves which is usually discarded in the preparation of chlorophyll, after thorough extraction with light petroleum to remove chlorophyll, fluoresced a quinine blue.

(2) *Oils.*

Certain oils were examined and were found to fluoresce blue. Cod liver oil gave a greenish yellow fluorescence. Table II shows an approximate comparison of the strength of blue fluorescence found in oils purchased locally.

Table II.

Oil	Dilution in ether fluorescing blue with intensity of 0·05 % quinine sulphate
Cotton seed	1 in 18
Linseed	1 in 18
Olive	1 in 3
Colza	1 in 5

In the case of the cotton seed oil it was found that the fluorescent constituent was present in the unsaponifiable fraction after removal of the main part of the cholesterol by digitonin. The others were not examined in this respect.

(3) *Other products.*

An extract prepared by Mr Peskett from cane sugar in the course of the preparation of bios-free cane sugar fluoresced blue.

It was found that a blue fluorescent extract could be obtained from sawdust by treatment with acid 50 % alcohol followed by addition of chloroform.

As mentioned in the introduction, yeast concentrates obtained by the charcoal method described elsewhere [Peters, 1924] fluoresced a quinine blue.

Some remarks upon the nature of the substances involved in the blue fluorescence.

In the main we confirm Bence Jones in that most animal tissues contain substances extractable in ether-alcohol mixtures which fluoresce blue under our conditions. We may call these "animal" quinoidine after Bence Jones, of which typical examples are the guano and urochrome fractions. The substance present passes into an alcohol-ether layer either directly or after saponification with alcoholic potash. There is however some difference in the behaviour of the fluorescent substance in gelatin, skin and the lens, which we may designate quinoidine "S." Fluorescence largely disappears upon saponification with alcoholic potash, and further cannot be made to pass into an ether layer. Whether this constitutes a real difference or depends upon some accidental association is not clear at present. The absence of blue fluorescence from blood plasma and serum[1], and also from egg albumin, shows that this fluorescence is not an inherent property of proteins, and the fact that crude gelatin fluoresces more powerfully than flocculated gelatin suggests that fluorescence of gelatin is due to an impurity.

The quinoidine fractions from urine and guano are very similar in appearance and behaviour. We think that the following arguments prove that blue fluorescence is not due to the presence of porphyrins: (1) As mentioned above, the traces of porphyrin present can be removed from the guano ether extracts without interfering with their blue fluorescence. (2) In the case of urine, porphyrins were shown to be present in normal urine in traces by Garrod [1893]. Owing to the work of H. Fischer and his colleagues we have to consider as possibilities uro-, copro- and Kammerer's porphyrin. We have never found that these porphyrins fluoresce blue. Specimens of uroporphyrin prepared by one of us from the urine of "Levi," a case of haematoporphyria congenita, by the methyl ester method of Fischer and Zerwecke [1924] fluoresce an intense red. Crude specimens of coproporphyrin show a yellowish red fluorescence. Turacin, believed by Fischer to be the copper salt of uroporphyrin did not fluoresce under our conditions. (3) The plant porphyrin fractions obtained by us showed no blue fluorescence. (4) The blue fluorescence is not due to the interaction of porphyrin and some other constituent because the addition of uroporphyrin to normal urine produces no intensification of the blue fluorescence, merely a change of hue.

Quinoidine extracts from guano have been obtained which gave no trace of Whitby's [1923] reaction for sterols with formaldehyde and sulphuric acid, and no blue colour with sulphuric acid alone, excluding sterols and lipochromes. Cholesterol itself was excluded by direct test of the pure substance (Table I). The Schlesinger test for urobilin was negative, also the Hymans van den Burgh reaction. Positive indican and kynurenic acid tests could not be obtained.

We feel that it is most probable that blue fluorescence may prove to be actually a property of urochrome for the following reasons. Firstly, the uroquinoidine extracts from guano are at least five times more fluorescent than quinine. If the fluorescence be merely due to contamination, there must be present a substance which is extraordinarily powerful as a source of blue fluorescence. Secondly, the behaviour of the aqueous fractions from guano and urine after saponification with alcoholic potash suggests the decomposition of some complex, which may quite possibly be of the glycuronic acid type. This would be consistent with the recent finding of Pollecoff [1924] that glycuronic acid is a constituent of urochrome. Two views have been taken about the origin of urochrome. The first is that of Roaf [1921] who considers that it is a derivative of chlorophyll, as he found that feeding upon the green parts of plants increases the amount of the urochrome fraction in the urine. The second is the view of Fischer and Zerwecke [1924], who have found evidence for the presence of certain amino acids in urochrome, and consider it to be a breakdown product of haemoglobin. Our evidence, based upon blue fluorescence, would suggest that urochrome comes from the green parts of plants, not from chlorophyll, but from some "blue fluorescent" substance present in the first acetone extract of the leaves.

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[1] See note, p. 406.

The fluorescence of "animal quinoidine" is fairly resistant to oxidation, but readily disappears upon treatment with acid solutions of nitrite.

Discussion.

There are two questions involved in this work. Firstly, do these substances fluoresce blue because of the possession of some common constituent? Further work will doubtless settle this point. Secondly, there is the broader biological question. What is the meaning of this widespread blue fluorescence which we find in animal epithelial structures, in the lens of the eye, in yeast, in sawdust and in grass? Whenever ultra-violet light falls upon these parts it is certain that they must be exhibiting this blue fluorescence. Up to the present it has been considered that the presence of fluorescent substances in the skin is dangerous to the animal organism. This view is based upon the experiments of Raab [1900], Tappeiner [1900], Hausmann [1911] and others upon the toxic effect of fluorescent substances, and upon the blistering found to occur in cases of haematoporphyria in sunlight. Are we to believe that, whereas the presence of a red fluorescence is dangerous, the presence of a blue fluorescence is not? In this connection the experiments of Suarez [1916] are of interest, who obtained from maize a substance resembling quinine in fluorescence, and called by Hofmeister "Zeochin." This zeochin sensitised blood corpuscles and organisms to the light, but when fed to animals in large amounts it did not render them light sensitive, thus suggesting that, normally, the animal can deal with such substances.

If "quinoidine" should turn out to be identical with urochrome, it would be of interest in connection with the work of Fischer and Zerwecke [1924]. They found that urochrome protected paramoecia against the toxic action of porphyrins in the light. It is difficult to believe that there is not some real function in this interaction of ultra-violet light with "quinoidine S." Recent experiments by Hess and Weinstock [1925], Steenbock, Black, Nelson, Nelson and Hoffert [1925] and Coward and Drummond [1925] show that irradiated cholesterol has antirachitic effect so that the presence of fluorescence cannot be essential to the formation of antirachitic substance. It may, however, be in some way directive. There is a "blue fluorescent" substance present in milk, sawdust and certain oils. Further, cotton seed and linseed oil are some six times more fluorescent than olive oil. It may be mere coincidence, but the facts seem to be related to the findings of Hess and Weinstock [1924] and Steenbock and Black [1924] that irradiated cotton seed and linseed oils give good cures whereas olive oil gives inconstant results.

SUMMARY.

1. The blue fluorescence of tissues in ultra-violet light (Å. 3000–4000) has been investigated qualitatively. Extracts fluorescing blue can be obtained, as was shown originally by Bence Jones who called the substance responsible "animal quinoidine."

2. Quinoidine extracts can be obtained from urine, guano, milk, feathers, liver and caseinogen, also from skin, commercial gelatin and the lens of the eye. The behaviour of the latter group to solvents is different. Whether this difference is the accident of some association is not yet clear.

3. Phytoquinoidines are present in grass leaves and certain oils, especially linseed and cotton seed, in yeast extracts and also in sawdust.

4. No pure substance among a number examined showed a quinine-like fluorescence. The fluorescence of porphyrins, chlorophyll, xanthophyll, and bile derivatives was not blue.

5. Upon fractionation of normal urine the quinoidine passes into the urochrome fraction (Garrod).

We are indebted to Sir Archibald Garrod and Dr Mackey for an introduction to the rare case of haematoporphyria congenita, to Professor Goodrich for turacin and to Sir F. G. Hopkins for a specimen of histidine. We are also most grateful to Professor Schryver for his kindness in sending specimens of flocculated gelatin. One of us (H. W. K.) is indebted to the Medical Research Council for a grant.

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LX. THE PECTIN CONTENT OF NORMAL AND "SILVERED" APPLE LEAVES.

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During a recent investigation the observation was made that the young bark of the apple contained a very appreciable amount of pectin. The variety of apple examined was Bramley's Seedling. The bark, after mincing, was thoroughly extracted with boiling alcohol and then, after drying, with boiling water. The aqueous extracts, after concentration under diminished pressure, yielded, on precipitation with alcohol, a product which appeared to be essentially identical with the pectin obtained from apple fruit, with the exception that it contained a very small amount of starch. On treatment with alkali it gave methyl alcohol and acetone and on treating its aqueous solution with the enzyme, pectase, a good gel was soon formed, although not quite so quickly as was the case when employing pectin obtained from the fruit.

This discovery of pectin in the young bark suggested that apple leaves might also contain this substance. A quantity of apple leaves was therefore examined for pectin in a manner similar to that employed when investigating the bark. A good yield of pectin was then obtained, but it was not quite so pure as that derived from the bark since it contained rather more starch and did not gel so quickly with pectase.

It was observed, however, that while the leaf fragments, which had been previously exhausted with boiling alcohol, were being deprived of pectin by means of boiling water they developed a silvery appearance precisely resembling that shown by leaves affected by the well-known "Silver Leaf" disease. The possibility of "Silver Leaf" being associated with pectin deficiency was therefore investigated.

As is well known, "Silver Leaf" is a disease particularly affecting plum trees, but other trees, including the apple, may also become infected by it. Leaves of the last mentioned were chosen for the present investigation since material particularly well adapted for this purpose was readily available, and, moreover, the results so obtained would be comparable with the observations already made, which are recorded above.

The tree chosen was an espalier apple of the "Keswick Codlin" variety, the upper branch on one side of which only was affected with "Silver Leaf" disease. On the morning of July 22nd 40 average leaves, without stalks, were collected from near the end of the diseased branch, and a like number from a similar position on the corresponding branch on the other side of the tree, which was perfectly normal. After weighing, each sample of leaves was cut into narrow strips and thrown into boiling alcohol. After boiling for 15 minutes the extractions were completed in a Soxhlet apparatus with fresh quantities of alcohol. After drying and weighing the material which had been exhausted with alcohol, each sample was extracted a number of times by boiling with large volumes of distilled water for the removal of the pectin. The latter, after concentration of the extracts under diminished pressure, was precipitated by means of alcohol, collected, dried and weighed. The results obtained are given below:

	Original weight of 40 leaves g.	Weight of alcohol-extracted marc g.	Weight of pectin g.	% of pectin in alcohol-extracted marc
Normal leaves	35·15	7·70	1·30	16·89
"Silvered" leaves	29·70	6·50	0·97	14·90

It is thus seen that the leaves affected by "Silver Leaf" disease contain an appreciably smaller amount of pectin than do normal leaves. Moreover, the yield of pectin calculated on the weight of the fresh, normal leaves (3·7 %) seems surprisingly large when compared with the proportion of this substance found by the present author [1921, 1923] to occur in the peel and pulp of the fresh fruit, namely, 2·6 and 1·3 % respectively. The pectin obtained from the leaves, however, is not so pure as that prepared from the fruit, but the amount of impurity present certainly does not seem sufficient to account for the increased yield obtained from the leaves as compared with that from the fruit.

Although the presence of pectin in apple leaves does not appear previously to have been recorded, it is known that they contain the enzyme, pectase. Thus, if fresh apple leaves be well pounded with a little water and the resulting mass strongly pressed, the juice so obtained, if added to a solution of pectin in the presence of chalk, causes the formation of a solid gel of calcium pectate. The expressed juice of the leaves appears, however, to contain little, if any, pectin.

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LXI. CHEMICAL INVESTIGATIONS OF FRUITS AND THEIR PRODUCTS.

I. APPLE JUICE AS A SOURCE OF SORBITOL.

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(*Received March 30th, 1925.*)

The material generally employed as a source of the hexahydric alcohol, sorbitol, namely, the berries of the Mountain Ash (*Pyrus aucuparia*, Gaertn.), is not always readily obtainable, even at the appropriate season of the year. Moreover, in the author's experience, it is by no means always an easy matter to isolate sorbitol from this source. During an investigation on the constituents of apples it has been found that they contain an appreciable proportion of sorbitol and, since apples are always obtainable and the isolation of sorbitol from them may easily be accomplished, it would appear that this fruit affords the most convenient source for the compound in question. The method employed for the isolation of sorbitol from apples is briefly as follows.

The apple juice was first concentrated under diminished pressure to about one-third its volume. A slight excess of basic lead acetate solution was then added and the resulting precipitate removed by filtration. The filtrate, after being deprived of lead by means of hydrogen sulphide, and the excess of the latter removed, was fermented with yeast in an incubator until the evolution of carbon dioxide had apparently ceased[1]. The mixture was then considerably concentrated under diminished pressure and again treated with basic lead acetate as previously described, after which the filtrate, which was now much diminished in volume, was again fermented, as it was found that an appreciable amount of sugar always remained after the first fermentation was apparently complete. A final clearing with lead was then employed and the filtrate, after being deprived of lead, was evaporated as far as possible under diminished pressure.

The gum-like residue so obtained was then covered with an excess of acetic anhydride, some pyridine added as a catalyst, and the mixture cautiously warmed on a water-bath, when acetylation occurred rapidly after the temperature had been somewhat raised. When the first vigorous reaction was over the mixture was kept boiling for 1 hour to ensure complete acetylation, after which it was cooled to about 50° and water cautiously added to decompose the excess of acetic anhydride present. Finally, a considerable excess of water was added and the mixture extracted several times with ether. The combined ethereal extracts were washed several times with water and then extracted successively with aqueous sodium carbonate and sodium hydroxide, after which they were again washed with water, and dried. During the extraction with alkalis and the final washing with water appreciable amounts of brown, resinous products were removed. On evaporating nearly all of the solvent from the purified ethereal extract a pale yellow residue was obtained, which soon crystallised in aggregates of hard, colourless prisms. The latter were deprived of adhering, pale yellow, viscid material by draining on a porous tile in an atmosphere saturated with ether. After recrystallisation from ethyl acetate colourless prisms melting sharply at 99° were obtained, and were identified as hexa-acetylsorbitol. In *Richter's Lexicon* the latter compound is described as a syrup, on the authority of Vincent and Delachanal [1889] but Thorpe [1912] gives the melting point as 99°, in accordance with the value found by the present author. Hexa-acetylsorbitol is readily soluble in alcohol, moderately so in ethyl acetate and rather sparingly soluble in ether. It dissolves slightly in boiling water.

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[1] The addition of some "yeast water" and a little ammonium phosphate greatly accelerates the fermentation.

For the regeneration of sorbitol 2 % aqueous sulphuric acid was added to a quantity of the above-described hexa-acetyl derivative and the mixture boiled in a flask attached to a reflux condenser until solution was complete. Steam was then passed through the mixture until acetic acid was no longer removed, after which the sulphuric acid was exactly precipitated by the addition of aqueous baryta, and the filtrate from the barium sulphate evaporated to dryness under diminished pressure. The residue so obtained was dissolved in alcohol from which it slowly separated in colourless crystals which, after drying, melted at 110°, and were identified as *d*-sorbitol.

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LXII. CHEMICAL INVESTIGATIONS OF FRUITS AND THEIR PRODUCTS.

II. THE FATE OF SUGAR DURING "CIDER SICKNESS."

By FRANK TUTIN.

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(*Received March 30th, 1925.*)

The disease known as "Cider Sickness" or "Second Fermentation" is, amongst all the disorders to which cider is liable, the most dreaded by cider makers in this country. It has been much studied by Barker [1906, 1907, 1911, 1912, 1, 2] who has shown that it is caused by a specific organism which attacks chiefly sweet ciders of low acidity when the temperature becomes somewhat raised. It causes a very rapid disappearance of the sugar, together with the formation of alcohol, acetaldehyde, formaldehyde, carbon dioxide, and hydrogen. He also obtained conclusive evidence that part of the sugar was converted into some non-reducing substance, which could not then be isolated nor its nature ascertained.

Since the acetylation method described in the preceding paper had proved so convenient for the isolation of sorbitol from apple juice it was decided to employ it for the investigation of the non-reducing substances present in "sick" cider. A quantity of the latter in which the "sickness" fermentation had been allowed to proceed to the ultimate limit was therefore concentrated under diminished pressure, purified by means of basic lead acetate, and the solids contained in the filtrate acetylated in the manner described in the foregoing paper. The resulting acetylated material, after purification in the manner employed when isolating the hexa-acetylsorbitol (preceding paper, p. 417), was obtained in noticeably greater yield than the corresponding product from the fresh juice. It could not, however, be induced to crystallise either by long keeping or by inoculating with a crystal of hexa-acetylsorbitol. When, however, a small particle of hexa-acetyl-*d*-mannitol was introduced crystallisation commenced and, during the course of some weeks, gradually spread throughout the viscid mass, but the proportion of crystalline material deposited was not large. The very viscid mixture was then brought on to a porous tile and allowed to drain in an atmosphere saturated with alcohol vapour. The white solid so obtained, after one crystallisation from alcohol, melted at 123° and was identified as hexa-acetyl-*d*-mannitol. A small amount of hexa-acetylsorbitol was subsequently isolated from the viscid material which had been absorbed by the tile.

Since no mannitol could be detected in apple juice after fermentation with yeast only, it appears evident that fermentation by the "sickness" bacillus results in the reduction of a portion of the sugar present to mannitol, a change which is known to be brought about by certain other bacteria and which is in harmony with the observation of Barker that hydrogen is evolved during "sickness" fermentation.

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LXIII. OXIDISING ENZYMES.

VIII. THE OXIDATION OF CERTAIN PARAHYDROXY-COMPOUNDS BY PLANT ENZYMES AND ITS CONNECTION WITH "TYROSINASE."

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(*Received April 1st, 1925.*)

It has been shown by one of us [Onslow, M. W., 1920] that the oxidases of the higher plants may be represented as a system consisting of two enzymes (oxygenase and peroxidase) and a substance having the orthodihydroxy-grouping of catechol. The oxygenase catalyses the autoxidation of catechol with formation of peroxide; the peroxidase catalyses the transference of active oxygen to guaiacum. Substances having the catechol grouping, for instance, catechol, protocatechuic acid, caffeic acid, hydrocaffeic acid and chlorogenic acid [Gorter, 1909] have been detected by various workers in a number of plants, all of which (in cases where tested) have been oxidase plants.

It was also shown [Onslow, M. W., 1920] that, in addition to substances with the catechol grouping, the widely distributed oxygenase will catalyse the oxidation of *p*-cresol with the resultant blueing of guaiacum. With this exception, however, the oxygenase was unable to act on various phenols with groupings other than catechol, for instance, phloroglucinol, resorcinol, etc. It was suggested [Onslow, M. W., 1923] that this behaviour of *p*-cresol might be explained by the presence of traces of a dihydroxy-derivative formed either by autoxidation or by traces of active oxygen in the enzyme preparation.

The present communication records the fact that when an enzyme preparation from the potato tuber acts on either *p*-cresol, tyrosol (*p*-hydroxyphenylethyl alcohol) or phenol a dihydroxy-derivative is formed which gives rise to peroxide and subsequent blueing of guaiacum.

The potato tuber, as previously shown, contains oxygenase, peroxidase and a substance with the catechol grouping. It also contains "tyrosinase" and free tyrosine or polypeptides of tyrosine, or both.

An enzyme preparation of the tuber was made by thoroughly extracting with cold alcohol, and then extracting the residue with water. When added to *p*-cresol solution, the mixture rapidly turned orange-pink. On addition of guaiacum to a portion, a deep blue colour was produced. On addition, to another portion, of a few drops of a weak solution of ferrous sulphate or ferrous ammonium sulphate, the pink colour turned green and subsequently with 1–2 % sodium carbonate blue, violet and red, a reaction characteristic of the catechol grouping. When the enzyme was added to tyrosol, a pink colour was rapidly developed. The solution then gave the same reactions as those of *p*-cresol with guaiacum and with ferrous sulphate and sodium carbonate. In the early (red) stage of the action of the enzyme on tyrosine the iron solution appeared to give the catechol reaction, but it was not normal.

The above results indicate a new type of oxidation. The solutions of *p*-cresol and tyrosol may contain traces of a dihydroxy-compound formed by autoxidation, too small in amount to be detected by the iron reaction[1]. Should this be so, the enzyme may be regarded as catalysing this autoxidation by means of active oxygen. An alternative suggestion is that the enzyme preparation is never quite free from traces of active oxygen which may produce traces of the dihydroxy-compound [Dakin and Herter, 1907] thus enabling the reaction to proceed. A third suggestion is that an enzyme exists which has a specific action on parahydroxy-compounds, and the same distribution as oxygenase.

An enzyme preparation from the tuber was also added to a solution of dihydroxyphenylalanine (prepared from *Vicia faba*). A red colour was first formed which eventually became black as in the case of tyrosine. Addition of guaiacum at the red stage did not produce more than a trace of blue colour on the surface of the liquid. It is well known, however, that the products of "tyrosinase" on tyrosine do not blue guaiacum.

It has been shown collectively by Chodat and Schweizer [1913], Happold and Raper [1925] and Robinson and McCance [1925] that *p*-cresol, catechol and phenol, in presence of an enzyme preparation from the potato tuber, will oxidise glycine, alanine and leucine.

One of us (M. E. R.) has also found that potato enzyme and tyrosol will oxidise glycine. Tyrosine and dihydroxyphenylalanine will not, however, effect the oxidation of glycine.

The conclusions which we draw from the above facts are as follows:

The oxygenase (or some other enzyme) oxidises *p*-cresol and tyrosol to 3.4-dihydroxytoluene and 3.4-dihydroxyphenylethyl alcohol respectively. These then give rise to active oxygen in the same way as catechol.

The active oxygen is the agent in the deamination of glycine, alanine and leucine.

In the case of dihydroxyphenylalanine, active oxygen formed may be used in deamination of the side chain. Hence there is no blueing of guaiacum. Dihydroxyphenylacetaldehyde and ammonia formed may take part in melanin formation.

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[1] Against this view in the case of *p*-cresol is the fact that a solution of this substance, on standing in air, does not give a blue colour with horse-radish peroxidase and guaiacum as does a solution of catechol kept under similar conditions.

Tyrosine may be oxidised (in the same way as tyrosol and *p*-cresol) to dihydroxyphenylalanine, the reaction proceeding subsequently as above.

On the other hand, Raper and Wormall [1925] maintain that deamination of tyrosine does not take place, since no ammonia can be detected during oxidation. In addition, they have stated that the enzyme has no action on *p*-hydroxyphenylpyruvic acid in the presence of ammonia.

It has been shown by Bach [1914] that the preliminary red colour formed in the oxidation of tyrosine by "tyrosinase" disappears if the products are placed under anaerobic conditions. Hence Bach postulated the presence in the potato of an oxido-reductase (deaminase) which forms products which are eventually oxidised to melanin either spontaneously or through the accelerating influence of a phenolase.

It has been suggested by one of us [Onslow, 1923] that the oxido-reductase may act as an intermediary between the active oxygen and the amino-acid.

Raper and Wormall [1923] doubt the existence of an oxido-reductase for the following reasons. Potato "tyrosinase" (dialysed juice) was allowed to act on tyrosine until a red colour was produced. The solution was divided into two portions. From one portion the enzymes were removed by colloidal "iron," leaving a red solution. Both portions were then placed in an incubator at 38°, and they decolorised equally well.

We noted, as did these authors, that the red oxidation product of "tyrosinase" on tyrosine was decolorised on heating. So also, to a lesser extent, was the red-brown product formed by the action of oxygenase on catechol. The same was true for the blue oxidation product of guaiacum. A similar experiment was made by us as follows. An enzyme preparation of the potato tuber was prepared by reprecipitation with alcohol so as to remove amino-acids as far as possible. This was allowed to act on catechol until a red-brown product was formed. Half the solution was treated with colloidal "iron." The two solutions, with and without enzyme, were then evacuated in Thunberg tubes and left at room temperature. The tube containing enzyme decolorised in 3 hours. The control tube remained coloured for one or more days. We do not necessarily conclude that an oxido-reductase is present, since the enzyme preparation would certainly contain proteolytic enzymes, and oxidised catechol is undoubtedly decolorised by products of autolysis.

Further work is in progress to determine, if possible, whether or no an oxido-reductase is present which catalyses the oxidation of the amino-acids present.

We conclude from the evidence available that the so-called action of "tyrosinase" may be as follows. Oxygenase (or some other enzyme) introduces a second hydroxy-grouping forming dihydroxyphenylalanine. Oxygenase catalyses the oxidation of this substance to a peroxide. Active oxygen may oxidise the amino grouping directly or may act as an acceptor for the hydrogen produced by an oxido-reductase, the amino-acid acting as the acceptor for the oxygen.

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Note added at proof reading, May 21*st*, 1925. We have found that *m*-cresol (both Merck and Kahlbaum samples) is oxidised by the enzyme preparation to a compound giving the catechol reaction. A 1–2 % solution of *p*-hydroxybenzoic acid, neutralised with sodium hydroxide to litmus, is also slowly oxidised by the enzyme, and the solution, after 24 hours, gives a slight but distinct catechol reaction.

LXIV. CHOLESTEROL SYNTHESIS IN THE ANIMAL BODY.

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(Received April 2nd, 1925.)

Ellis and Gardner [1909] attempted to determine whether cholesterol synthesis occurred in the animal body by determining the amount of cholesterol in a number of unincubated eggs and in newly-born chicks from the same batch of eggs. They found that the amount in the newly-born chicks did not differ from that in the eggs, and they concluded that cholesterol synthesis did not occur. They laboured however under the difficulty that they were using a method of cholesterol estimation much less accurate than that available at the present time. Gardner and Lander [1914] approached the question from another point of view. They estimated the cholesterol in a number of newly-born chicks and fed the remainder of these chicks on various diets—chicken food, chicken food previously freed from sterols, and chicken food plus 2 % cholesterol, and, after 7, 14 and 21 days, further estimations were made on individuals from the various groups. Owing to irregular rate of growth and consequent irregular weights, the results were not very definite, but the authors conclude that cholesterol synthesis does not take place. Lander [1915], carrying out similar experiments on rats, found that the percentage cholesterol was, on the average, 161 mg. at 3 weeks of age. He fed other rats on mixed, and in some cases ether-extracted, diets, but none of his animals lived for any length of time owing to the fact that his diets were deficient in vitamin A. His inconclusive results are therefore open to objection, but he expresses the view that "there is no evidence that the living organism can manufacture cholesterol, but on the other hand, it is strictly conserved and readily picked out from the diet." His method of extracting the cholesterol was to mince the animals, grind them up with plaster of Paris, and extract the powdered material for 14 days with ether. Cholesterol was estimated in the extract by the digitonin method.

The work so far described has had as its basis the estimation of cholesterol in the whole body of the animals under experiment. Another method has been to measure the total sterol intake on mixed diets and the total excretion in the faeces. Thus Gardner and Fox [1921], working with eight adults on mixed diets, found that the faecal sterol output was about 2·5 times greater than the intake. In all cases save one this large excess excretion over intake was maintained, and they conclude that there is some organ in the body capable of synthesising cholesterol. Similar results are reported by Gamble and Blackfan [1920] in which the sterol output was 1·3–3·4 times the intake. These concordant results lead the authors to conclude that cholesterol synthesis takes place, but there are certain facts which this type of experiment ignores. Thus, even though the sterol output is greater than the intake, no rigid conclusion as to cholesterol synthesis can be drawn unless knowledge exists as to the function of the cholesterol stored in the various organs of the body. Little is known on this subject and the excretion of cholesterol might possibly ensue as the result of some physiological function; in this case if the diet were poor in cholesterol, the reserve supplies in the liver, adrenals, etc., might be called upon to make up the deficiency, and hence, unless experiments of this type were carried out over a prolonged period, the possibility of the effect being due to cholesterol already stored in the body cannot be eliminated. A second objection is that bacterial synthesis of sterol in the large intestine may be the cause of the large excess of excretion over intake. It was with a view to investigating these possible sources of error that the work described in this paper was undertaken. The general plan was to estimate the amount of cholesterol in rats at various ages and weights when on the complete artificial diet suggested by Drummond and Coward [1920] and used in this laboratory, in others on a diet poor in cholesterol, and in others still on a diet completely free from cholesterol.

The difficulty with regard to the cholesterol-free diet has hitherto been that no diet has been devised which is free from cholesterol and yet sufficient for growth. The ether-extracted diets used by other workers have necessarily been free from fat and therefore from fat-soluble vitamins. Hence where such diets have been used the results have suffered from the fact that growth, if any, has been very slight, and in experiments of this type it would appear essential that the animals should exhibit normal growth throughout. This difficulty has been overcome in the present work by supplying the rats with a very small daily dose of the unsaponifiable matter from cod liver oil. This is prepared by extracting cod liver oil, after saponification with alcoholic potash, with ether. After washing the extract and removing the ether, the resulting product, which still contains about 5 % of oil, is again saponified and extracted with ether as before. The washed extract, after drying over anhydrous Na_2SO_4, is taken to dryness. The unsaponifiable matter so obtained contains about half its weight of cholesterol, the bulk of which can be removed by crystallisation from 95 % methyl alcohol. After filtration and removal of the solvent, the residue is dissolved in boiling 95 % ethyl alcohol and the remaining cholesterol precipitated by excess of a 1 % solution of digitonin in 90 % alcohol. After filtration and removal of the excess digitonin, the solvent is evaporated off. Suitable precautions are taken to prevent oxidation throughout the preparation.

Diets used in the experiment.

I. Complete artificial diet	%	II. Cholesterol-free diet	%	III. Diet poor in cholesterol	%
Caseinogen	20	Caseinogen (ether extracted)	20	Caseinogen (ether extracted)	20
Rice starch	50	Rice starch	68	Rice starch	68
Butter	15	Butter	0	Butter	0
Yeast extract	5	Yeast extract	7	Yeast extract	7
Lemon juice	5	Lemon juice	0	Lemon juice	0
Salt mixture	5	Salt mixture	5	Salt mixture	5
		Unsap. matter (cholesterol-free)	0·0005 g. per rat per day	Unsap. matter	0·0005 g. per rat per day
				Cholesterol	0·004 g.

With a view to economising the unsaponifiable matter, it was dissolved in synthetic triolein and each animal of the group on the cholesterol-free diet received one drop of this solution per day, this being made up so that one drop contained approximately 0·0005 g. unsaponifiable matter. The cholesterol of diet III was given in a similar manner.

Fat and cholesterol content of the diets.

In the cholesterol-free diet (II), the fat (butter) was omitted and the starch increased to 68 %. The lemon juice was also omitted, as rats appear to be able to grow satisfactorily on a diet deficient in vitamin C. In order to render the diet more palatable, the percentage of yeast extract was increased to 7. As the caseinogen used had been thoroughly extracted, this diet was virtually fat-free. To obtain its fat content, 25 g., after grinding with anhydrous Na_2SO_4 were extracted with ether for 40 hours, and yielded 0·2 % ether-soluble material. This was saponified with sodium ethylate and the resulting unsaponifiable matter gave no precipitate with digitonin and none of the cholesterol colour reactions. As further confirmation that the diet was quite free from cholesterol, 25 g. were saponified first with aqueous and then with alcoholic potash. The unsaponifiable matter obtained by ether extraction also failed to give any of the cholesterol colour reactions; this diet was thus cholesterol-free, and constituted the basis of diets II and III. In diet III, each animal received three drops of a 4 % solution of cholesterol in triolein, thus receiving approximately 4 mg. cholesterol per day.

Diet	Basal fat g. per rat per day	Fat in supplement g. per rat per day	Total fat g. per rat per day	Total cholesterol g. per rat per day
I. Complete artificial	5·5	—	—	0·0015
II. Cholesterol-free	0·02	0·025	0·045	—
III. Poor in cholesterol	0·02	0·075	0·095	0·004

Animals used in the experiment.

All the rats used in this work were bred from a definite number of our own stock, with a view to eliminating abnormal results as far as possible. The original plan was to estimate the cholesterol content of rats at time of weaning (age, 3–4 weeks, weight, 35–45 g.) and to take an average of the figures obtained as representing the normal cholesterol content of rats at this age and weight. Any animals put on to any diet at weaning were thus considered as starting with this average amount of cholesterol. Later it was decided to do estimations on numbers of rats on the complete artificial diet (diet I) at various ages and weights—in other words to obtain figures for a curve of cholesterol content of rats on this diet plotted against age or weight. This was to serve as a control of the results obtained from animals on the other diets. Every group of animals on any given diet was a mixture from the various litters, care being taken that the litters were well scattered among the different groups in order to obtain more average results.

Method of estimation.

Each rat was killed by chloroform; its alimentary canal was removed from the stomach downwards and washed through with water until quite empty. In this way only the cholesterol present in the tissues was estimated. The animal, together with the washed stomach, intestine, etc., was then placed in a flask with sufficient 10 % KOH and boiled for 3–5 hours, when complete solution, save of the bone fragments, had taken place. It was considered that in this way only could all the cholesterol be freed from the tissues. The results, which will be reported later, show a higher cholesterol content than those of Lander [1915] for animals of the same age and weight. This would suggest that either the cholesterol was not completely extracted by his method or else that his animals were considerably under the weight which those reared in this laboratory normally show at the same age. After saponification by aqueous KOH, the liquid was extracted with ether six times in a separating funnel after addition of sufficient alcohol to cause rapid separation of the ether and aqueous layers. The extract—usually about 500 cc.—was washed five times with water, and after removal of the ether the resulting fat was dried at 105° for half an hour. It was then boiled for 2 hours with sodium ethylate ($2N$) after solution in hot alcohol. Water was then added to dissolve the soap and the solution was extracted with ether and treated as already described for the previous saponification. The ether was then removed in a tared flask and the whole dried for 1 hour at 105° and re-weighed. In this way the weight of unsaponifiable matter in each rat was obtained. The cholesterol present in this unsaponifiable matter was then estimated by the method of Windaus [1910] in an aliquot part of a solution in light petroleum in order to economise digitonin. The fraction of the solution taken was always such that it contained, as far as possible, the same weight of unsaponifiable matter. After removal of the light petroleum the unsaponifiable matter was dissolved in 10 cc. 95 % ethyl alcohol and precipitated in boiling solution with 0·2 g. digitonin in 90 % alcohol. In this way the weight of cholesterol digitonide weighed was kept fairly constant. After standing over night the precipitate was collected in a Gooch crucible, washed first with ether and then with boiling water until free from digitonin, dried for 1½ hours at 105° and weighed. From the weight of the digitonide, the total cholesterol in each animal can be readily calculated. The method is tedious, but ensures that all the cholesterol present in the animal is estimated. Owing to the large number of manipulative operations, great care is necessary; further, any error in the actual estimation of the cholesterol is greatly increased owing to the amount of unsaponifiable matter taken being only a fraction of the whole. The results obtained are taken as representing the amount of cholesterol present although the possible occurrence of other sterols precipitable by digitonin must be kept in sight.

Results.

1. *Complete artificial diet.*

	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat (pairs) g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	11·0	16·4	11·8	20·6	11	25·6
	10·9	17·4	12·0	21·0	11	26·8
	11·3	17·2	12·1	20·3		
	9·6	14·8	13·2	22·9		
	10·4	17·8	13·0	20·4		
			12·9	21·6		
Mean	5·3	8·4	6·2	10·6	11	26·2

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	23·1	67·3	27·5	73	34·3	76
	20·8	57·5	24·0	69	34·1	71
	22·0	56·3	30·1	64	36·3	74
	21·5	58·3	27·0	86	34·8	73
	19·2	52·3	29·0	88	38·0	98
	21·2	50·3	30·3	78	38·0	103
	19·4	43·9			35·0	92
					35·0	87
					34·0	86
					33·2	98
					31·5	88
					32·5	92
					31·3	85
					38·0	71
					40·0	69
					37·6	71
Mean	21	55	28	76	35	83

	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.	Wt. of rat g.	Cholesterol mg.
	68	134	83	141	109	238	137	262
	64	122	100	161	114	183	140	282
	65	110	100	167	110	179	139	262
	70	143	90	163	111	195	140	268
	59	142	90	194	110	197	155	288
	57	141	100	162	121	263	155	279
	63	131	94	165	122	210	152	299
					105	212	148	280
					113	187	146	278
					112	229		
					113	209		
Mean	64	132	94	165	113	208	146	278

2. *Cholesterol content of* 100 *g. rats receiving the cholesterol-free diet.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	42	103	225	219
	42	102	237	232
	46	98	219	223
	47	103	225	218
	42	102	227	224
	47	107	230	214
	41	107	226	210
	41	102	230	225
	37	100	224	224
	37	97	215	222
	50	94	214	229
	37	98	213	217
Mean	42	101	224	221

3. *Cholesterol content of* 100 *g. rats receiving cholesterol-free diet plus* 4 *mg. cholesterol per rat per day.*

	Weight when weaned and put on to diet g.	Final weight when used for estimation g.	Cholesterol mg.	Cholesterol mg. per 100 g. rat
	47	113	227	214
	37	106	243	225
	54	108	241	213
	55	110	241	213
	48	108	246	228
	56	110	238	216
Mean	49	109	240	218

It is recognised that there are objections to taking averages of figures such as these but the variations are not as great as might be expected considering the biological nature of the experiment, that the animals used were from many litters and that the ages of animals of similar weight often varied. It would have been an advantage if the number of animals used for estimation had been greater, but this was limited by the difficulty of raising sufficient litters from the limited number of stock animals selected for breeding for this experiment, and also by the tedious nature of each estimation. However, from the average figures it is possible to construct the curve in Fig. 1 in which the abscissae give the weights of the rat in g. and the ordinates the cholesterol content in mg. From this curve it will be seen that the cholesterol content of the young rat at weaning time is about 100 mg. and this figure is taken as the approximate content of all the other animals put into the other groups.

It would appear from this curve that a linear relationship exists between cholesterol content and body weight; further, the curve may be divided into two parts, one from birth to a weight of about 35 g., and a second part which is less steep, and it would appear that the increase of cholesterol with increase of body weight is definitely more marked in the early stages. This is shown more clearly by the curve in Fig. 2 in which are plotted as abscissae the ages of the animals and as ordinates the amount of cholesterol expressed as mg. per 100 g. body weight.

It will be observed that there is a rapid increase in the percentage cholesterol to an age of 3 weeks followed by a fall to a value slightly above that at birth. The figures from which this curve has been constructed have been obtained by the conversion of some of the figures in the previous table to cholesterol percentages and their rearrangement into groups of animals of the same age. This was not possible with all, as records of all the ages wer not kept. This curve is much less regular than the preceding one—a not unexpected result—but it would seem to indicate that the maximum cholesterol percentage corresponds approximately to the time of weaning at the age of 3 weeks, and this would suggest that it was due to completion of construction of nervous tissue. One other possibility which may explain this rise to a maximum is that the digestive tracts of the animals which were killed before weaning were not removed before estimation of the cholesterol content, and hence they may have retained in it some sterol from their mother's milk, but it is difficult to explain the results on this fact alone.

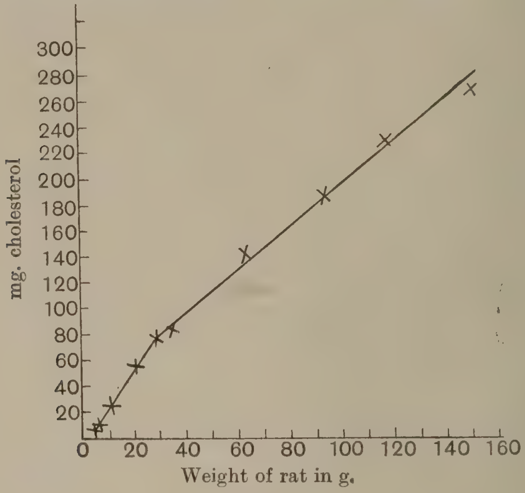

Fig. 1.

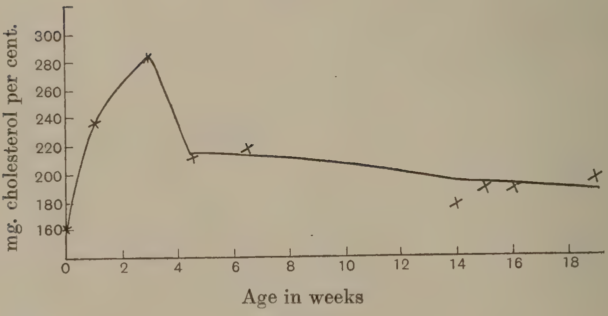

Fig. 2.

Cholesterol content of rats on the cholesterol-free diet at approximate weight of 100 *g.*

From an initial average weight of 42 g. to a final average weight of 101 g., the cholesterol has increased from 100 mg. (obtained from the curve in Fig. 1) to 224 mg. Using the figures reduced to 100 g. body weight, it is seen that the ratios of the final to initial body weights, and of the final to initial cholesterol contents are 100/42 and 221/100, *i.e.* 2·4 and 2·2 respectively; in other words, the rate of increase in cholesterol content has been very nearly as rapid as that of body weight. As the animals received no cholesterol over this period of 6 weeks during which their average weight rose from 42 to 100 g. and as their cholesterol content rose from 100 to 221 mg., an increase of 0·121 g. per animal, it would seem clear that cholesterol has been synthesised.

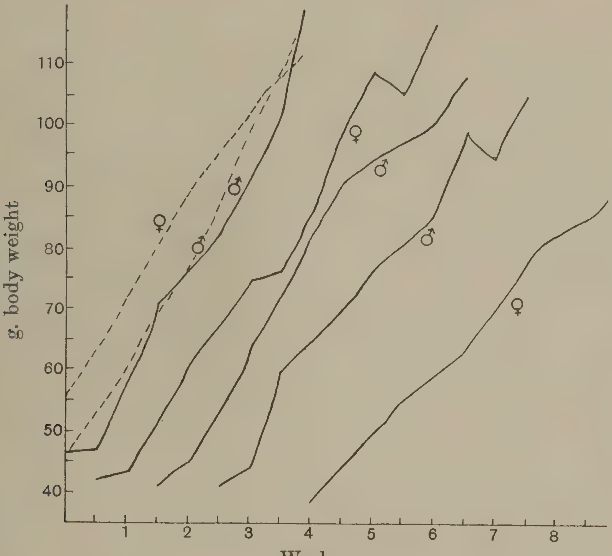

Fig. 3.

That the growth of the animals on the cholesterol-free diet was quite normal is shown in Fig. 3 in which are given the growth curves of six animals receiving this diet and taken at random. For comparison, there are given as dotted lines what are considered in this laboratory to be normal curves for animals receiving a diet complete in every respect.

Each animal received during the 6 weeks of diet III approximately 170 mg. of cholesterol, and had it all been stored, the total content of the animals which already had 100 mg. at the beginning, would have been 270 mg. even if synthesis had not occurred. It is curious that the average figure per 100 g. body weight in this group is 219 mg., whereas of those receiving no cholesterol (group 2) the average figure is 221 mg., the ingestion of 170 mg. having made no difference to the final cholesterol content of the animals of group 3. It is to be observed that in group 1 (complete artificial diet) the cholesterol content of 100 g. rat is about 190 mg. whereas in both groups 2 and 3 the figure is about 220. No explanation of this difference has been obtained.

Sterol excretion.

Cholesterol was estimated in the ether extract of the dried faeces after 72 hours' extraction. The average figure per animal over a weekly period on the cholesterol-free diet was 0·0198 g. which would give for the 6 weeks of the experiment a total of 0·119 g. On diet III (poor in cholesterol) the excretion for the whole period was 0·126 g. per animal.

Thus the total sterol synthesised (that in the tissues was 0·121 g.) was 0·240 g. by each animal in 6 weeks.

SUMMARY.

1. A curve showing the relation between the body weight of rats from birth to 150 g. weight and the cholesterol content has been constructed for animals reared on the complete artificial diet used in this laboratory.

2. Rats fed from weaning time until they had reached 100 g. weight on a cholesterol-free diet showed an increase in cholesterol content from 100–220 mg.; the animal body would thus seem capable of synthesising cholesterol.

The writer wishes to thank Professor J. C. Drummond for his kindly help and suggestions, and the Government Grant Committee of the Royal Society for a grant from which part of the expenses of this research were defrayed.

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LXV. SOME PHOSPHORUS COMPOUNDS OF MILK.

I. THE PRESENCE IN MILK OF ORGANIC ACID-SOLUBLE PHOSPHORUS COMPOUNDS.

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Milk, as the sole foodstuff of the majority of young, growing mammals, contains all the substances necessary for this growth—proteins, fats, carbohydrates, salts and certain as yet chemically unknown factors. The necessity for further knowledge concerning these unknown substances is evident and urgent, and the present communication deals with the earlier stages of an investigation of the organic acid-soluble phosphorus compounds which are to be found in protein-free milk filtrates.

My attention was first drawn to these substances during the course of an enquiry into the nature of the acid-soluble phosphorus compounds of blood [Kay and Robison, 1924, 1, 2], when it was found that blood corpuscles contained at least two such esters, both hydrolysed by acids. One was hydrolysed by extracts of bone and of other tissues, whilst the other was more refractory towards enzyme activity. An average of 30 % of the organic acid-soluble phosphorus of blood was present in the more readily hydrolysed form. The view that these esters play an important part in bone formation has been supported by much evidence brought forward by Robison and his co-workers, and their possible rôle in carbohydrate metabolism [Kay and Robison, 1924, 2] and in the acute acidosis which follows ammonium chloride ingestion [Kay, 1924] has also received attention. The likelihood that young animals might receive their organic phosphorus direct from the milk, though, as experiments shortly to be described will indicate, a somewhat remote one, led me at the time to search for similar substances in milk. It was then found that phosphorus compounds, very similar to those occurring in blood corpuscles, were present in a free state in fresh milk in important quantities.

Although during the last 30 years a certain amount of intermittent attention has been paid to the organic phosphorus compounds of milk other than caseinogen, little is known as to the actual chemical state of the phosphorus, and still less has light been thrown on to its possible biochemical significance. It has been accepted for some time, in spite of sporadic denials with regard to the last two forms, that phosphorus occurs in organic combination in milk in at least three forms—as caseinogen, as lecithin, and also in protein-free, lecithin-free milk filtrates. The work of Osborne and Wakeman [1915, 1916] on the phosphatides of milk has established their presence and identity firmly, one having been shown to be lecithin, another being found to be a diaminomonophosphatide. But we still do not know how the phosphate group is united to the rest of the molecule in caseinogen, nor whether there is any biological necessity for the presence of this group in the molecule of the protein. Our knowledge of the acid-soluble organic phosphorus compounds is, up to the present, hardly more than nominal. Apart from having given names which are somewhat misleading to these compounds the earlier workers can only be said to have rather shakily established their presence.

It is not surprising that discussion should have arisen as to the bare fact of the existence in milk of compounds belonging to the last group when one reviews the earlier methods used for getting rid of the other milk constituents. Siegfried [1896], for example, succeeded in separating a phosphorus-containing, non-protein substance from milk by precipitating the caseinogen with acetic acid and removing the albumin and globulin by boiling the filtrate. He then precipitated the free phosphates with calcium chloride and ammonia, and boiled the filtrate with excess of ferric chloride and ammonia. The precipitate formed contained iron, phosphorus and nitrogen, and was of roughly constant composition. It was variously called "nucleon," "carniferrin" or "phosphorfleischsaüre," the last two because it was considered to be allied in composition to a substance obtained from muscle, using a similar technique. Wittmaack [1897] went even further. He precipitated the caseinogen from human milk by warming the diluted milk to 50° with dilute hydrochloric acid, then boiled the filtrate to coagulate the albumin and globulin, and examined the protein-free filtrate for "nucleon." Sikes' [1906] criticism is not without justification, that this treatment hardly errs on the side of caution, and that any "phosphocarnic acid" found in the later stages might well be produced during this drastic process of protein precipitation. He instances Bayliss and Plimmer's [1905] finding that part of the caseinogen phosphorus may readily be split off in organic, non-protein form. Sikes asserted that no "phosphocarnic acid" was present in the milk he was investigating—human milk—when the protein was precipitated with tannic acid mixture in the cold, though he gave no experimental details to support his statement.

There is, however, little doubt that organic phosphorus compounds are present in the filtrates from cow's, goat's and human milk from which caseinogen and other proteins have been carefully removed, as the experiments outlined in this communication abundantly show. Methods of protein precipitation have been employed which are not open to the above criticism, and further, when more than one method has been applied to the same milk sample, the quantitative results have agreed well.

(It is of interest to note that "lecithin" in milk has passed through the same stages of assertion [Nerking and Haensel, 1908], denial [Njegoram, 1913] and re-establishment [Osborne and Wakeman, 1915].)

Cow's, goat's and human milk have been investigated, and a readily hydrolysable organic phosphoric ester has been found present in all samples examined. For the normal milk of each species there is a fairly constant amount of this ester present, but the quantity differs from one species to another. It varies somewhat during the period of lactation, being greatest in goat's milk in the colostrum, and slowly diminishing in the later stages. (This aspect is still under investigation, and will not be dealt with here.) That these amounts are by no means negligible may be the more readily appreciated when it is pointed out that, in the case of human and of goat's milk, the readily hydrolysable phosphoric ester accounts on the average for between 10 % and 20 % of the total milk phosphorus, including that of caseinogen. In cow's milk the proportion of hydrolysable phosphorus is about 5 %.

EXPERIMENTAL.

In the earlier experiments fresh milk was obtained and kept as cool as possible until the proteins had been precipitated. The fact that the similar hydrolysable organic phosphorus of blood diminishes on standing, particularly if the blood be laked, owing to the activity of a phosphoric esterase in the corpuscles, suggested that a similar enzyme might also be present in milk, but it was found later that there was no appreciable diminution in the amount of readily hydrolysable phosphorus in milk after it had been allowed to stand for some hours at room temperature, or even in presence of antiseptic at 38°.

Methods of removal of proteins.

The first method used was to precipitate the caseinogen by bringing the diluted milk to the isoelectric point of this protein with dilute acetic acid, and then to throw down the albumin and globulin from the filtrate by adding sufficient trichloroacetic acid solution to make a final concentration of this acid of 2·5 %. The neutralised filtrate was used for the phosphorus determinations.

The second method, and the one usually employed in the later experiments, was to carry out the whole of the precipitation in one operation by the use of "dialysed iron." A perfectly clear, iron-free filtrate, giving no biuret reaction, and containing very nearly as much hydrolysable phosphoric ester as the filtrate prepared by the first method, could thus be obtained. The filtrate from the dialysed iron precipitation contained, however, much less free phosphate and was therefore more useful for determinations of increase of this ion. This diminution in free phosphate is the result of two causes: (1) caseinogen takes down with it the minimum of free phosphate if it is precipitated in the neighbourhood of its isoelectric point (p_H 4·7) [György, 1923], whilst the dialysed iron precipitate was well on the alkaline side of this, and (2) there was some formation of insoluble ferric phosphate.

Using the same sample of milk in each case and the phosphoric esterase of bone as the hydrolytic agent the following results were obtained for the quantities of hydrolysable phosphoric ester present.

Table I. *Hydrolysable phosphorus in mg. per* 100 *cc. milk.*

	Proteins precipitated by acetic and trichloroacetic acids	Proteins precipitated by dialysed iron
Cow's milk	4·6	4·2
	4·7	4·9
	5·0	4·5
Average	4·77	4·53
Goat's milk	17·1	17·1
,, colostrum	25·4	23·0

From the above figures it appears possible that in addition to most of the inorganic, a small fraction of the hydrolysable organic phosphorus may have been carried down by the dialysed iron, but this, for present purposes, is insignificant.

In order to get complete precipitation of the proteins from milk with dialysed iron, certain precautions are advisable. It is better to use quantities of milk which are not too large, and the following volumes for normal goat's milk work well. 30 cc. milk are diluted to 120 cc. with distilled water. 45 cc. of 1·5 % dialysed iron (prepared by diluting B.P. *liquor ferri dialysati*) are run slowly from a separating funnel into the diluted milk, which is held preferably in a stout bottle to allow of continuous and fairly violent shaking. After the whole 45 cc. has been introduced, the bottle is shaken well and allowed to stand 10 minutes. The filtrate is usually perfectly clear. Rather less dialysed iron is required for 30 cc. cow's milk, usually from 35–40 cc. being sufficient[1].

The filtrate is now made faintly pink to phenolphthalein with a few drops of $N/10$ NaOH, and allowed to stand. If a precipitate appears it is filtered off (it does not contain organic phosphorus). The filtrate is now ready for the action of enzymes or other agents.

The method employed throughout for the determination of free phosphate was that of Briggs. A comparison between the results obtained by this method, and those obtained by the Neumann method as adopted for micro-estimations by Robison [1923] using the same milk filtrate, is given below. The hydrolysing agent used was a 5 % extract of rat bone. The figures represent mg. free phosphorus per 50 cc. of milk filtrate.

Table II.

	At 0 hours		After 18 hours at 38°		After 40 hours at 38°	
	Briggs	Neumann	Briggs	Neumann	Briggs	Neumann
A. Cow's milk filtrate + enzyme	0·34	0·33	0·67	0·64	0·75	0·70
B. Cow's milk filtrate + boiled enzyme	0·34	0·32	—	—	0·34	0·30
C. Cow's milk filtrate + water	0·24	0·23	—	—	0·25	0·25

The amount of enzyme used in this experiment was only half that used in the later determinations reported in this paper, in which the hydrolysis was completed, or very nearly so, in 18 hours.

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[1] It has been found that the commercial "B.P." dialysed iron varies considerably in precipitating power and in acidity. The figures given above are therefore no more than an approximate guide.

The discrepancy, which is not large, between the values found by the two methods is doubtless due to the fact that a small amount of protein from the enzyme solution was present in A and B and interfered with the complete precipitation of the ammonium phosphomolybdate in the micro-Neumann method. Traces of this compound remained in colloidal solution and were thus not estimated. It is quite clear, however, from these figures that there is no analytical fallacy in applying the Briggs method to such milk filtrates.

Amounts of hydrolysable phosphoric ester present.

The amount of this ester present was determined by adding 5 % extract of rat bone to the neutralised milk filtrate in sufficient quantity to complete the hydrolysis in 18 hours, determining the free phosphate at zero hour and at intervals. It is convenient to use the bone enzyme, as some of its capabilities are known from previous work with blood filtrates, but, as will be seen, extract of intestinal mucosa, or of kidney will, under similar conditions, effect the same hydrolysis. In certain cases the last two extracts have been found to hydrolyse slightly more than bone extract, so that the figures for enzyme-hydrolysable ester when bone extract is used are to be taken as minimal.

Method.

10 cc. of the milk filtrate is measured into each of a series of test tubes, and 1·0 cc. 5 % bone extract and 0·25 cc. chloroform are added. Boiled enzyme replaces the active enzyme in the control tubes. The whole series, carefully stoppered, is then placed in the incubator at 38°. At intervals, two tubes are taken, and 2 cc. 15 % trichloroacetic acid are added to each, to precipitate the enzyme proteins. Of the filtrate from each tube, 10 cc. are taken for a Briggs' phosphate determination. The duplicates usually agree exactly. The results of a typical experiment are given below.

Table III. *Goat's milk, precipitated by dialysed iron.*

	mg. phosphorus per 100 cc. milk			
	0 hours	6 hours	18 hours	24 hours
Free P	7·9	24·1	24·5	24·5
Hydrolysed P	—	16·2	16·6	16·6

It will be observed that most of the hydrolysis takes place in the first 6 hours.

Table IV gives the collected results of a number of determinations using the milk of three different species.

It is clear that goat's milk contains considerably more phosphoric ester than either cow's milk or human milk, and in most of the experiments yet to be described, goat's milk has been used as the source of the ester. The relative constancy of the values shown above for cow's milk is probably due to the fact that each sample contained the mixed milk of several cows. The figure for "non-hydrolysable" phosphorus is the difference between the total acid-soluble phosphorus in the milk filtrate (determined by the usual incineration method) and the sum of the inorganic and hydrolysable phosphorus.

Table IV. *Mg. organic acid-soluble phosphorus per* 100 *cc. milk.*

Cow's milk		Human milk		Goat's milk		Goat's colostrum
Hydrolysed by bone enzyme	Not hydrolysed	Hydrolysed	Not hydrolysed	Hydrolysed	Not hydrolysed	Hydrolysed
4·6	2·9	3·9*	2·0	16·6	—	25·4†
4·7	1·0	3·1*	2·0	17·1†	—	23·0†
5·0	3·6	2·5*	1·4	17·1†	—	
4·2	—	4·0	—	12·0	3·2	
4·9	—	2·4	2·0	8·6†	4·4	
2·5	2·1	3·1	4·0	8·9†	—	
4·5	1·1	[<0·5	—]	16·5	1·5	
4·4	3·5	[1·2	2·9]	12·4	1·7	
		[1·0	2·3]	10·6	—	
		4·0	1·9			
		3·1	4·0			
		3·2	2·8			
		3·3	3·9			
Av. 4·35	2·3	3·25	2·5	13·3	2·7	

* These samples late in lactation period from different mothers.
† Same sample, using different methods of precipitating proteins.

Note.—The three human milk samples in brackets are not included in the averages as they are probably abnormal. The first of the three was obtained from a mother whose child had just died from intestinal trouble, the last two were from hospital cases of difficult labour.

A comparison of these average amounts with those obtained for "nucleon" or "phosphocarnic acid" phosphorus by previous workers is of interest, although, as stated earlier, some of their methods would hardly be expected to give reliable figures. A short calculation from the published figures of Siegfried [1896], Wittmaack [1897] and Ellenberger, Seeliger and Klimmer [1902] has been made.

Table V.

mg. P per 100 cc. milk.

	Nucleon or Phosphorfleischsaüre P			Hydrolysable P	Total organic acid-soluble P
	Siegfried	Wittmaack	E. S. & K.	(Present author)	
Cow's milk	3·9	3·4	4·7	4·4	6·7
Goat's milk	—	8·7	—	13·3	16·0
Human milk	8·7	8·5	9·5	3·3	5·8

Only in the case of cow's milk is there approximate agreement. It would seem that there is some, but not a very direct or quantitative correspondence between the substances which have been called "nucleon" and the organic, phosphorus-containing substances present in milk filtrates, and estimated by the methods described in this paper.

The absence from milk of a phosphoric esterase.

Different samples of milk from animals of the same species, particularly goat's milk, gave markedly different figures for the hydrolysable phosphorus.

It was at first thought that this might be due to the presence of an enzyme in the milk itself.

No such enzyme appears to be present. Several experiments have been made (1) allowing the milk to stand for some hours at laboratory temperature, (2) allowing the milk to stand at 38° for 24 hours in presence of antiseptic, and in no case were more than minimal quantities of organic acid-soluble phosphorus hydrolysed. The results of two experiments are summarised in Tables VI and VII.

Table VI. *Effect of standing at room temperature.*

mg. P per 100 cc. milk.

		P hydrolysed by bone enzyme at 38°			
	Free P	After 6 hours	After 18 hours	Total acid-soluble P	Non-hydrolysed acid-soluble P
Milk drawn into cold bottle and precipitated at once	7·9	16·5	16·5	25·9	1·5
Same milk stood 4 hours at 20°	7·9	16·2	16·6	25·6	1·1
Same milk stood 8 hours at 20°	7·8	16·2	16·2	25·6	1·6
Stood overnight at 0°	8·8	16·2	16·4	26·8	1·6

Table VII. *Effect of standing at* 38°.

mg. P per 100 cc. milk.

	P hydrolysed by bone enzyme at 38°	
	After 6 hours	After 24 hours
Fresh milk precipitated at once	9·9	10·6
Same milk after standing 6 hours at 38°	9·4	10·1
Same milk after standing 24 hours at 38°	8·4	9·5
Same milk boiled for 1 minute	9·9	10·5
Boiled milk after standing 24 hours at 38°	8·8	9·9

If a phosphoric esterase is present at all in fresh milk it is there in no more than traces. Boiling for 1 minute appears not to affect the organic acid-soluble phosphorus of fresh milk.

The rate of hydrolysis of the acid-soluble phosphorus compounds by bone enzyme and by acid.

The rate of hydrolysis by bone enzyme was first determined in order to find out when hydrolysis could be taken as completed. Later, filtrates from the three kinds of milk examined were compared. The results, together with the curve given by a blood filtrate under similar conditions of hydrolysis [Kay and Robison, 1924, 1], are shown in Fig. 1.

In each case, using bone enzyme, well over 80 % of the hydrolysis occurs in the first 6 hours. Using double quantities of enzyme, the reaction goes to completion in this time so that determinations of the amount of hydrolysable phosphorus in a given sample of milk can be made in a single day.

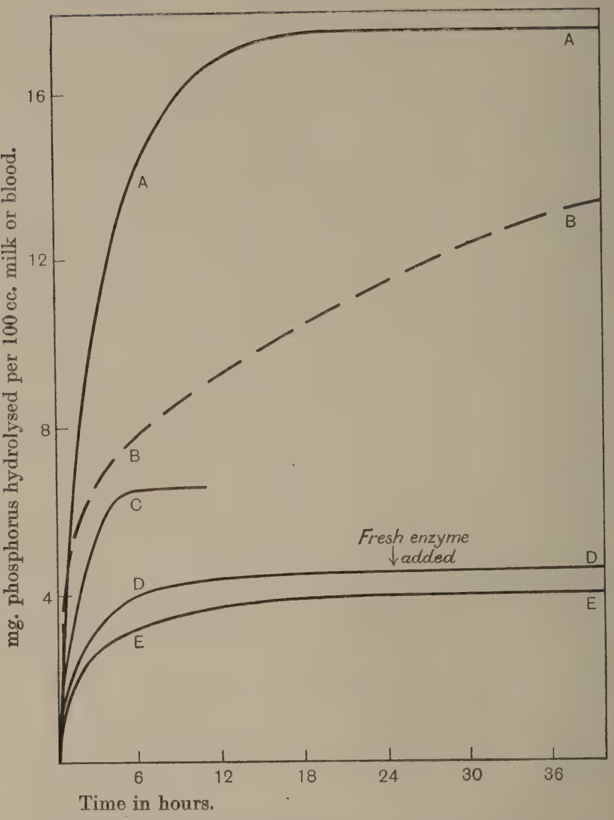

Fig. 1. A. Goat's milk filtrate + bone enzyme at 38°.
B. Goat's milk filtrate + $N/4$ sulphuric acid at 100°.
C. Rabbit's blood filtrate + bone enzyme at 38°.
D. Cow's milk filtrate + bone enzyme at 38°.
E. Human milk filtrate + bone enzyme at 38°.
Strength of enzyme: 1 cc. 5 % bone extract to each 20 cc. filtrate.

Action of various reagents on the phosphorus compounds.

The substrate is more stable to heat, acids and alkalies than its ready hydrolysis by bone enzyme would suggest. Results of experiments are summarised below.

Milk filtrate + antiseptic kept at 0° at p_H 8·2	Less than 5 % change in 3 months
Filtrate at p_H 8·2 boiled for 2 minutes	Less than 3 % hydrolysis
Prolonged boiling at p_H 8·2	Very slow hydrolysis
Autoclaving 1 hour at 120° (p_H 8·2)	11 % hydrolysis
$2N/3$ sulphuric acid at 38°	No hydrolysis in 18 hours
Autoclaving 1 hour with $N/10$ sulphuric acid at 120°	25 % hydrolysis
Boiling ½ hour $N/4$ sulphuric acid	22 % hydrolysis
Boiling 48 hours $N/4$ sulphuric acid	80 % hydrolysis
Heating with $2N$ sulphuric acid for 72 hours at 100°	Hydrolysis not yet complete
$N/2$ sodium hydroxide at 38°	Slight hydrolysis after 18 hours
$N/2$ sodium hydroxide at 100°	Marked hydrolysis in ½ hour and 80 % hydrolysis after 18 hours

The proportion of organic phosphorus which remains unhydrolysed after prolonged boiling with dilute sulphuric acid varies with the milk sample, and agrees in general with the findings in the bone enzyme hydrolyses, that a portion of the organic phosphorus, varying irregularly from 10–40 % of the total is not hydrolysable.

Plimmer [1913] found that glycerophosphates and similar esters containing PO_4 in the same grouping were quite stable to N alkali at 95°. The instability of the hydrolysable phosphoric ester, at least towards alkali, would indicate that it is not of the glycerophosphate type.

Action of digestive enzymes and tissue extracts.

In order to obtain information as to the possibility of the absorption of these milk compounds unchanged from the alimentary tract, a few experiments have been carried out on the hydrolytic power of digestive enzymes *in vitro* at 38°. At the same time it was thought worth while to investigate the distribution in various tissues of a phosphoric esterase similar to that present in bone. The results are summarised below.

Ptyalin. Very active ptyalin gave absolutely no hydrolysis, either at p_H 7 or at p_H 8·4 in 24 hours.

Pepsin. Armour's pepsin, very active towards fibrin, had no action whatever on the organic phosphorus of two milk filtrates at the optimum p_H for this enzyme.

Trypsin. Active trypsin (Liquor Trypsin Co., Allen and Hanbury), rapidly hydrolysing fibrin and caseinogen, was entirely without action on the organic phosphorus, either at p_H 7 or p_H 8·4.

Pancreatic juice, obtained by cannula direct from the duct of an anaesthetised dog, but not activated, had a slight but definite hydrolysing action. The same juice, activated by adding a 10 % extract of duodenal mucosa, hydrolysed as much organic phosphorus as did the bone enzyme. The effect was traced to the intestinal extract itself, which was able to hydrolyse the same, or even a slightly greater amount of the organic phosphorus than the bone enzyme.

Extract of pancreas and of spleen. A 5 % pancreatic extract from an adult dog gave in 18 hours almost as much hydrolysis (7·8 mg. P per 100 cc. milk) at p_H 8·4 as did bone enzyme (9·0 mg. P), whilst 5 % spleen extract was almost inactive (0·9 mg. P).

Intestinal extract. A 5 % extract of duodenal mucosa gave the same amount of hydrolysis as standard bone extract:

Table VIII.

mg. P per 100 cc. milk. Initial p_H 8·4. Time 18 hours.

Experiment	Hydrolysed duodenal extract	Hydrolysed bone extract
A	16·4	16·5
B	16·6	16·6
C	12·6	12·0
D	9·0	8·8

Intestinal juice. The juice was obtained by drawing the contents of the small intestine of a starving dog through cotton wool. The clear fluid so obtained was diluted to give a 5 % solution. At p_H 8·4 the diluted juice of two adult dogs gave in each case the same amount of hydrolysis as with bone enzyme. A fortnight-old puppy's diluted juice gave 28 %, a 7 days old puppy's 66 % of the amount hydrolysed by bone enzyme in the same time.

Gastric mucosa. A 5 % extract of the gastric mucosa of a dog brought about, at p_H 8·4, 75 % of the hydrolysis effected by bone enzyme. It was thus somewhat less active than intestinal mucosa.

Kidney extract. 5 % extract was slightly more active than bone enzyme at p_H 8·2, hydrolysing, in one experiment, as much as 16 % more organic phosphorus.

Mammary gland. A 10 % extract of the mammary gland of a lactating goat hydrolysed at p_H 8·4, 10·1 mg. P per 100 cc. milk in 24 hours, whilst standard bone enzyme in the same time hydrolysed 12·9 mg. P. The same mammary extract, when added to a protein-free filtrate from rabbit's blood, hydrolysed 5·9 mg. P %, whilst bone enzyme in the same time hydrolysed 9·2 mg. P %.

Blood. Diluted blood (one part blood to four of water) gave in 48 hours a small but definite amount of hydrolysis.

Muscle extracts. Fresh rabbit muscle extract, made by grinding up the tissue with sand and 10 times its weight of chloroform water and filtering, brought about, at p_H 8·4, 15 % hydrolysis of the organic phosphorus after 11 hours, and 22 % hydrolysis after 48 hours. By this time the hydrolysis had virtually ceased.

If the hydrolysable phosphorus compounds had been esters of the hexosediphosphate type, this concentration of muscle enzyme and substrate should have resulted in hydrolysis of the order of 52 % in 11 hours, and 82 % in 48 hours [Kay and Robison, 1924, 2, Tables I and II]. Thus there is indirect evidence that these compounds are probably not to any large extent esters of the yeast hexosediphosphate type. As in blood, so in milk there would appear to be at least three forms of organic acid-soluble phosphorus present, the portion hydrolysable by bone enzyme in each case containing more than one substance. Consideration of the rate of hydrolysis of the organic phosphorus of milk filtrate by boiling with $N/4$ sulphuric acid leads to a similar view, the velocity constant falling off rapidly, and then becoming constant at about 8 hours, remaining constant until the experiment is discontinued at 48 hours.

Collecting the foregoing results into one table:

Table IX.

	Activity		Activity
Ptyalin	0	Intestinal extract	+ + + +
Pepsin	0	Spleen extract	+
Trypsin	0	Kidney	+ + + +
Pancreatic juice	+	Muscle	+ +
Intestinal juice	+ + +	Mammary gland	+ + +
Gastric mucosa	+ + +	Blood	+
Pancreas extract	+ + +	Bone extract	+ + + +

The effect of dialysis.

Raudnitz [1903], in criticising some of the "nucleon" findings mentioned earlier in the present paper, says that so long as it cannot be found in milk dialysates, the pre-existence of nucleon in milk is doubtful. The acid-soluble phosphorus compounds of milk may readily be separated from the milk colloids by dialysis at low temperatures in collodion sacs, and there would appear to be no reason to believe that they are not present in the same form in fresh milk. The results of a dialysis experiment are given below.

Table X.

mg. P per 100 cc. fresh goat's milk. Dialysis at 0°.

	In filtrate after pptn. of orig. milk with dialysed iron	Dialysate	
		6 hours	22 hours
Phosphate P	2·5*	11·4	12·8
Total organic acid-soluble P	13·0	11·2	12·0
Hydrolysable P	8·6	5·1	6·1
Non-hydrolysable P	4·4*	6·1	5·9

* The dialysed iron taken down much of the phosphate P and probably also some of the non-hydrolysable P.

It is clear that both types of organic phosphorus may be separated from goat's milk by dialysis. The experiment has been repeated using cow's milk, and the same result obtained.

DISCUSSION.

The presence of acid-soluble, organic phosphorus compounds in milk having been established, questions arise as to the origin and function of these compounds.

They must originate either (1) by diffusion from the red blood corpuscle in which very similar compounds are present, or (2) by synthesis in the mammary gland, or (3) by breakdown of the cells of the mammary gland with discharge of their nuclear materials and other contents into the milk, or (4) by the breakdown of some more complex organic compound after secretion of the milk. The last possibility seems to be excluded by experiments mentioned early in this paper, the third is open to the objection that since, at least in goat's and in human milk, the amount of the phosphorus of the acid-soluble compounds is a relatively large fraction (15–25 %) of the total phosphorus of the milk, their derivation from this source would indicate a far more rapid breakdown of glandular tissue than appears possible.

That these phosphorus compounds may leak from the blood into the milk, as for example, urea and creatine [Denis, Talbot and Minot, 1919] appear to do, seems at first unlikely, since the phosphoric esters of blood are carried *in vivo* almost entirely in the corpuscles. It is possible, however, that when the corpuscles are slowly squeezing through the smaller capillaries, and are in intimate contact over a large portion of their surface with the capillary wall, conditions may exist for a direct interchange of substances between them and the cells of the capillaries[1]. One would at least expect fundamental alteration in the surface relationships. As against the possibility of such diffusion from the blood is the fact that the bone-enzyme-hydrolysable phosphorus compound is preponderant in milk, whereas in blood it forms only one-third, or thereabouts, of the total phosphoric esters. This is, perhaps, not a very serious objection, since all recent work in this field goes to show that the phosphorus compounds in the tissues have a far more intimate kinetic relationship with one another than was previously realised, cf. the findings of Lawaczeck [1924] and of Hansman, Martland and Robison [1924] working on the dynamics of phosphoric acid compounds in blood.

The remaining possibility, that the substance may be definitely manufactured and secreted by the cells of the mammary gland derives some little support from the fact that an enzyme is present in mammary glands capable, *in vitro*, of hydrolysis of part of the acid-soluble phosphorus compounds, and possibly associated, *in vivo*, with their synthesis.

As regards the further history and possible function of these compounds, from the experiments with digestive enzymes and extracts of alimentary mucosa just described, it would seem on the whole unlikely that the organic phosphorus compounds are absorbed unchanged from the milk. Nevertheless, they are not attacked by the digestive enzymes before their arrival in the intestine, and in view of their relatively rapid diffusibility, there is at least the possibility of their being absorbed unchanged.

Table XI.

Forms in which phosphorus occurs in milk.

1. Free phosphate.
2. Phosphate loosely associated with caseinogen and not diffusible [György, 1923].
3. Caseinogen phosphorus.
4. Lecithin.
5. Diaminomonophosphatide [Osborne and Wakeman, 1915, 1916].
6. Acid-soluble phosphoric ester, hydrolysed by muscle phosphatase.
7. Acid-soluble phosphoric ester hydrolysed by bone enzyme, etc.
8. Acid-soluble organic phosphorus compound unaffected by these enzymes and stable to acids.

To the present author it would appear not unlikely that the imperative necessity for meeting the phosphorus requirements of the growing suckling has led, in the mammary gland, to the conscription of every possible synthetic mechanism by which the phosphorus content of the milk can be increased. Moreover, assuming that complete hydrolysis actually does precede absorption, and remembering that phosphorus compounds occur in milk in at least seven other forms of combination beside that of caseinogen (see Table XI), there is the supplementary possibility that this series has been evolved in relation to certain solubility and absorptive difficulties in the intestine. Having differing hydrolysability and therefore, during digestion, maintaining for a longer or shorter period their un-ionised PO_4, these compounds may be absorbed, with or without previous hydrolysis, in different forms, and at different levels in the small intestine, so that both calcium and phosphorus, each essential to the growing suckling, may be taken up most advantageously. This could be achieved if the concentration of PO_4''' ions were kept continuously low, so that the solubility product of $Ca_3(PO_4)_2$ should not be exceeded, and as little transformation as possible into this almost insoluble salt—and therefore the loss of both its irreplaceable constituents in the faeces—ensue.

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[1] This conception may perhaps also be extended to the hypothesis advanced by Robison and his co-workers, that the organic esters in the red corpuscles play an important part in bone formation. A serious difficulty of this hypothesis has been the fact that at most only very small amounts of these esters are present, under normal conditions, in the plasma.

The chemical identity of these substances is now being investigated, and from preliminary experiments it appears that one of the organic acid-soluble phosphorus compounds present in goat's milk filtrate, and hydrolysable by bone enzyme, is also capable of reducing alkaline copper solutions.

SUMMARY.

It has been found that at least two acid-soluble compounds containing phosphorus in organic combination are present in cow's, goat's and human milk, one compound being readily hydrolysable by extracts of various tissues and by dilute acids, and one being considerably more stable.

The more readily hydrolysable compound is present to the average extent of 4·4 mg. P per 100 cc. in cow's milk, 13·3 mg. in goat's milk, and 3·3 mg. in human milk. In the last two milks, the acid-soluble organic phosphorus compounds account for from 10–25 % of the total phosphorus of the milk.

Both the hydrolysable and the stable phosphorus compounds pass readily through a collodion dialysing membrane which holds back protein but allows methylene blue to diffuse slowly.

Though not acted on by pepsin or trypsin, and only slowly attacked by pancreatic juice, the hydrolysable ester is probably broken down by the intestinal juice before absorption. Extracts of many tissues are also able to hydrolyse this phosphoric ester.

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LXVI. NOTE ON THE PHOSPHORUS CONTENT OF THE BLOOD OF RUMINANTS.

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(*Received April 3rd, 1925.*)

From the foregoing communication it will be seen that goat's milk contains a relatively large proportion of readily hydrolysable phosphoric ester. The experiments recorded below were begun in order to find out whether the phosphorus distribution in goat's blood could be in any way correlated with the unusual milk phosphorus distribution.

Abderhalden [1897, 1898] showed that goat's blood, in common with that of the other ruminants he examined (cow and sheep), contained much less total phosphorus than did the blood of the horse, the pig, the rabbit, the dog and the cat. His figures, collected from both these papers and recalculated in terms of milligrams P per 100 cc. blood, are as follows:

Table I.

	Sheep's blood		Ox blood	Bull's blood	Goat's blood	Pig's blood
	I	II				
Inorganic P	8·3	6·3	7·5	7·6	6·0	32·7
Total P	18·0	17·1	17·6	17·1	17·3	43·9
Lecithin P	8·9	9·6	9·4	8·8	9·8	9·2
Organic acid-soluble P	0·8	1·2	0·7	0·7	1·5	2·0
Acid-soluble P (Total – lecithin)	9·1	7·5	8·2	8·3	7·5	34·7

	Rabbit's blood	Dog's blood		Cat's blood	Horse's blood	
		I	II		I	II
Inorganic P	29·9	25·1	25·4	23·8	35·2	35·2
Total P	43·0	35·3	35·5	36·2	48·9	49·3
Lecithin P	11·3	8·2	8·0	9·3	11·6	11·9
Organic acid-soluble P	1·8	2·0	2·1	3·1	2·1	2·2
Acid-soluble P (Total – lecithin)	31·7	27·1	27·5	26·9	37·3	37·4

These figures show clearly the quantitative difference in the *total* phosphorus between the various animals, but at the time the analyses were performed it was not realised how easily autolysable was the organic phosphorus of the blood, and in consequence all the above inorganic phosphorus figures are much too high, and the organic phosphorus figures much too low.

It seemed of interest to determine whether the paucity of acid-soluble phosphorus in the blood of ruminants was due to lack of the "hydrolysable" or the "non-hydrolysable" organic phosphorus compounds reported by Kay and Robison [1924] as being present in the blood of rats, rabbits, and man. If, for example, that fraction of the organic phosphorus which is hydrolysed by bone enzyme were completely absent from the blood of ruminants, the view that the organic phosphorus compounds of the hexosephosphoric ester type in the blood are directly concerned with ossification would lose some of its cogency. Actually, however, the main shortage of phosphorus, as compared with the blood of rodents or of man, is in the fraction that is *not* hydrolysable by bone enzyme.

Blood was taken in the slaughterhouse direct into oxalate, and was brought at once to the laboratory and the proteins precipitated in the usual way. Inorganic phosphorus was determined by the Briggs method; total acid-soluble phosphorus by ignition with a sulphuric-nitric acid mixture followed by the same colorimetric procedure; lecithin phosphorus by the method of Randles and Knudson [1922] and the hydrolysable phosphorus by Kay and Robison's method [1924, p. 757] with slight modifications.

The results are shown below. Average figures for the phosphorus distribution in human, rabbit's and rat's blood are appended for comparison.

Table II.

	P in mg. per 100 cc. whole blood						% red corpuscles haema-tocrit	P in mg. per 100 cc. red corpuscles	
Animal	Total acid-soluble	In-organic	Organic	Hydro-lysable	Non-hy-drolysable	Lecithin P		Hydro-lysable	Non-hy-drolysable
Goat 1*	9·8	6·9	2·9	2·2	0·7	—	30	7·3	2·3
„ 2	7·6	4·0	3·6	2·5	1·1	7·5	30·5	8·1	3·6
„ 3	9·8	5·9	3·9	2·9	1·0	—	40	7·2	2·5
„ 4*	9·2	5·4	3·8	3·5	0·3	—	—	—	0·9§
„ 5	9·5	6·1	3·4	3·1	0·3	8·8	34	9·1	0·9
„ 6	9·2	5·9	3·3	3·0	0·3	—	35	8·6	0·9
Av. for 6 goats	9·2	5·7	3·5	2·9	0·6	—	34	8·1	1·8
Sheep 1	10·9	5·9	5·0	3·0	2·0	10·7	40	7·5	5·0
„ 2	11·4	5·7	5·7	3·6	2·1	9·9	39	9·2	5·4
„ 3	12·1	6·9	5·2	3·0	2·2	9·7	38·5	7·8	5·7
Av. for 3 sheep	11·5	6·2	5·3	3·2	2·1	10·1	39	8·2	5·4
Bullock 1	10·0	5·6	4·4	2·5	1·9	7·4	44	5·7	4·3
Cow	12·4	7·2	5·3	2·6	2·6	10·9	43	6·0	6·0
Bullock 2	9·4	4·9	4·5	3·0	1·5	10·9	49	6·1	3·1
Av. for 3 cattle	10·6	5·9	4·7	2·7	2·0	9·7	45	5·9	4·4
Man†. Av. for 5 men	25·8	3·1	22·7	6·4	16·3	11·8	43	14·9	38·0
Rabbit†. Av. for 10 adults	34·5	5·7	28·8	8·7	20·1	10·8‡	33	26·4	61·0
Rat†. Av. for 5 groups of adults	31·5	5·4	26·1	6·2	19·9	—	—	—	—

* Lactating.
† Taken from Kay and Robison [1924, Table I] and from hitherto unpublished figures.
‡ Average of 7 adults.
§ Assuming the average value of 34 % red corpuscles.

Table II indicates even more definitely than Table I the difference between the phosphorus partition in the blood of ruminants and in that of the three other species for which reliable figures can be given. Goats, in particular, have practically no non-hydrolysable organic phosphorus, either in whole blood or in the corpuscles, whilst sheep and cattle have only small amounts. The quantity of phosphoric ester hydrolysed by bone enzyme is low for all three ruminants, being about one-half of that in the blood of the other species. What the metabolic significance of these extraordinary differences between the blood of ruminants and of other mammals may be awaits further experiment, but as far as classification is concerned it would appear from the evidence at present available that the unusual blood-phosphorus content is characteristic of the tribe *Ruminantia* only, and not of the whole of the sub-order *Artiodactyla*.

If the organic acid-soluble phosphorus compounds in goat's milk (average 16·0 mg. P per 100 cc. milk) come from the similar compounds in the blood of these animals (average 3·5 mg. per 100 cc. blood), there must be active concentration of this phosphorus going on in the mammary gland. The more likely hypothesis, in view of these figures, is that the milk compounds, at least to a large extent, are synthesised in the mammary gland.

With the micro-methods now available, the determination of the P/N ratio in blood or in extracted blood-stains affords a simple chemical test which might be of use in medico-legal investigations for distinguishing the blood of sheep, cattle and goats from human blood.

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LXVII. THE INFLUENCE OF WASHING UPON THE REPRODUCTIVE RATE OF *COLPIDIUM COLPODA.*

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(*Received April 4th, 1925.*)

In a recent paper Robertson [1924, 1] reports that he has observed allelocatalysis in cultures of *Colpidium* sp. both in hay infusions and in synthetic medium. He also explains our earlier failure to obtain this effect in cultures of *Colpidium colpoda* [Cutler and Crump, 1923] as being due to too great a concentration of the parent culture medium in the sub-cultures (a dilution of from 1/37–1/980), and claims that if the organisms had been washed until the dilution was considerably greater (1/9000 in his own case) the allelocatalytic effect would have been observed.

This is of course a direct contraversion of certain facts published by this author in 1923 [Robertson, 1923, p. 97], which demonstrated that the allelocatalytic effect occurs in sub-cultures in which large quantities of "autocatalyst" derived from the parent must have been present. "Into a drop, measuring about 0·08 cc. of freshly prepared hay infusion was isolated an infusorian (*Enchelys*) derived from a densely inhabited parent-culture which was 7 days old. At the same time two separately isolated individuals from the same parent-culture were introduced together into a similar drop of infusion. The following results were obtained:"

Culture number	Number of individuals initially introduced	Number of individuals	
		After 24 hours	After 48 hours
310 A	1	2	16
311 A	2	3	120

Here, if we may take it that the individuals were isolated into drops of a similar size to those used in other experiments, namely, 0·002–0·003 cc., the dilution is only 1 in 40, and moreover the parent culture had been the scene of very active reproduction.

Under similar conditions we have demonstrated that there is no trace of mutual acceleration in individuals of *Colpidium colpoda*, as is shown in Table I, compiled from the data given in a previous paper [Cutler and Crump, 1923]. The cells in question were isolated from a 2 days old culture in which there had been rapid reproduction and they were grown, in the usual way, in chambers containing synthetic medium.

Table I. *Reproductive rate after 48 hours in cultures in which the dilution of the parent culture liquid in the sub-culture is less than* 1 *in* 200.

	Reproductive rates	Average
One animal	2·0, 2·0, 5·1, 3·8	3·2
Two animals	3·6, 1·0, 2·4, 3·5	2·6

The present paper gives the results of a series of experiments on *Colpidium colpoda* in which the organisms were washed before isolation thereby reducing the concentration of autocatalyst carried over from the parent culture to an amount comparable with that in Robertson's experiments. The washing was effected by centrifuging a culture of known ancestry, *i.e.* age of culture and amount of reproduction which had occurred, siphoning off the supernatant liquid, and adding fresh medium to the remaining 1 cc., and repeating this process until a suitable dilution was reached. From the final liquid the animals were isolated into a capillary tube with about 0·2 mm.^{3} of fluid, and inoculated into a chamber containing a quantity of medium varying from 1·6–9·2 mm.^{3}, in which bacteria from a 1 day old agar slope had been emulsified. The dilution of the parent culture varies in these experiments from 1 in 2540 to 1 in 22,900.

It is necessary to point out that where a small quantity of liquid containing rapidly moving organisms is delivered from a fine capillary tube it does not follow that the same number of organisms will always be contained in every drop, hence one or two organisms from the same parent culture can be obtained in drops whose sizes vary only within very narrow limits.

Table II shows the results obtained in sub-cultures from 1 and 2 animals which have been washed before inoculation.

Table II. *Reproductive rate after* 24 *hours' growth of washed individuals derived from young parent cultures* (24–48 *hours old*).

Number originally isolated	Degree of dilution of fluid from parent culture in liquid of sub-culture		
	1 in 1,000–5,000	1 in 5,000–10,000	1 in 10,000–20,000
1	4·0, 4·1, 3·3, 4·1, 4·7, 3·3, 3·0	4·8, 3·0, 3·0, 3·0, 4·0, 3·0, 4·0, 4·0, 4·1, 4·6, 4·0	3·5, 3·3, 4·2, 3·8, 4·0, 4·0, 4·0, 4·0, 4·6, 4·0, 4·0, 4·1, 4·2, 4·6
Average	3·8	3·8	4·2
2	3·9, 3·8, 4·2, 4·2, 3·3, 3·9, 2·4, 3·6	3·3, 2·5, 3·0, 4·0, 3·3, 2·0, 2·9, 4·0, 3·9	3·5, 3·9, 3·0, 4·3, 4·2, 3·9, 4·2, 3·7, 3·8, 3·9, 3·7, 3·2, 3·9
Average	3·7	3·2	3·8

After 48 hours' growth the averages for the same series of cultures are:

One animal	7·0	6·6	7·4
Two animals	5·6	6·1	6·6

It is obvious from the figures that in *Colpidium colpoda* allelocatalysis does not occur under the conditions in which Robertson has detected it both in *Enchelys* and in the other species of ciliate which he doubtfully assigns to the genus Colpidium. His description and figures of the animal, however

[Robertson, 1924, 2], indicate that he was not dealing with any species of the genus *Colpidium*, but more probably with one of the *Colpoda* genus.

The fact that this series of experiments shows higher reproductive rates at 24 hours than those given in our earlier paper [Cutler and Crump, 1923, 2], *e.g.* 3·8 as against 2·4, is due to the fact that in this series the cultures were fed with an unidentified species of bacterium [Cutler and Crump, 1924] which gives uniformly better results than are obtained where the cultures are treated with *Sarcina* as was the case in the earlier experiments.

To support his contention Robertson has also re-grouped some of the figures given in our earlier paper [Cutler and Crump, 1924] on *Colpidium colpoda*, and from these derives further support for his theory that the reproductive rate is retarded by a high concentration of the substances introduced with the organisms from the parent culture, because retardation appears to occur in the cultures of smaller volume into which the individuals were introduced. Column 1 of Table III shows the figures quoted by Robertson; the other columns he omitted in his paper. The numbers given are, however, all averages of individual reproductive rates and statistical treatment shows that the variation between the individual reproductive rates in any one group is as great as it is between those of different groups; and that therefore the apparent rise in the average reproductive rate from the smaller to the larger drops is a matter of pure chance; in short, there is no significant difference between any of the numbers in the original table.

Table III.

Vol. of substance in cu. mm.	Average reproductive rate in first 24 hours			
	One animal	Two animals	Three animals	Four animals
0–1·5	1·98	2·49	2·39	2·31
1·5–3·0	2·27	2·29	2·36	2·46
3·0–6·0	2·86	3·75*	2·46	2·50
6·0–9·0	2·87	2·84	(No cases)	3·30*

* Only two cases.

A further difference between the results obtained by Robertson and ourselves lies in the effect of washing on the organisms. Robertson [1924, 2] records that in his *Colpidium* sp. abnormal forms were sometimes produced in cultures from washed individuals, but in the case of our organism abnormal animals have never been seen. He has further found that washing affects the division of his species in that the animals in cultures from unwashed individuals tend to divide into four, those from individuals that have been washed once into either two or four, and those from individuals washed two or three times into two only. Again in the case of *Colpidium colpoda* this difficulty in the study of reproductive rates does not occur since reproduction in this species is by binary fission only, and washing does not retard the rate of reproduction as is shown in Table IV.

Table IV. *Average reproductive rates in cultures from washed and unwashed individuals.*

Initial inoculum	Unwashed. Dilution of parent liquid less than 1 in 35	Washed. Dilution of parent liquid 1/5,000–1/10,000	1/10,000–1/200
One animal (after 24 hours)	4·45	3·75	3·86
Two animals (after 24 hours)	3·10	3·55	3·67
One animal (after 48 hours)	7·35	6·67	7·52
Two animals (after 48 hours)	6·75	5·70	6·55

SUMMARY.

In *Colpidium colpoda* allelocatalysis has not been observed in cultures either from washed or unwashed individuals.

Washing the animals has no deleterious effect upon them nor does it retard the reproductive rate.

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LXVIII. THE CHEMICAL NATURE OF THE MEMBRANE OF POTATO CORK.

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Introduction.

In all the vascular plants a very important part is played by cell walls the physical properties of which have been altered by deposition of fatty substances in and upon them. As these bodies modify considerably the penetrability of the wall to water and aqueous solutions it is very essential to have information as to the nature of the fatty deposit, its continuity, permanence and physical properties. Such information is gradually being accumulated in this laboratory, but a much fuller knowledge is required of the chemical nature of the fatty bodies involved than is at present available and the present paper is a first contribution to that end in the case of cork.

In the shoot of the flowering plant there are two very different types of fat-impregnated membranes. From its earliest appearance the shoot is clothed with a cuticle, a fatty layer which is continuous over the whole surface of the shoot, as a deposit upon the outer walls of the outermost layer of cells. Frequently in older stems this layer is replaced in later life by a layer of cork which has quite a different origin. In this case a layer of cells, always lying below the actual epidermal cell, enters into a stage of active division and growth. As a result, a series of cells are cut off to the outside of this meristematic cell of which the oldest is the outermost (Fig. 1 A). Within each of these cells there now forms an inner layer which possesses fat-staining properties and which is called the suberin lamella. Similar fatty substances impregnate the thin wall medianly placed between two adjacent cells and known as the middle lamella, but the continuous suberin lamella within the cell is laid down simultaneously in all parts of the cell and undoubtedly is formed from the contents of the cell itself which have accumulated at the surface of the protoplast (Fig. 1).

Within this suberin lamella is present a layer of cellulose impregnated to some extent with fat but quite recognisable when all fatty bodies are removed. The fatty substances which are under investigation therefore in cork are not in a continuous sheet, as in the cuticle, but mainly concentrated within a series of isolated lamellae lining individual cells, the suberin lamellae, and also to some extent diffused through a carbohydrate basis of cellulose or of pectates in the middle lamellae.

A brief historical account of our chemical knowledge of suberin has been given by Priestley [1921]. The basis of the assumption that it is fatty in nature is its behaviour towards fat stains such as Sudan III and Scharlach R. On the other hand, neutral fat solvents only remove a small proportion of these substances [Gilson, 1890] of which only a small fraction has been shown to be glycerides of fatty acids [Kügler, 1884]. The rest of the material of the suberin lamella is removed by boiling in alcoholic potassium hydroxide [Van Wisselingh, 1886–1895], and this extract is usually regarded as consisting in the main of salts of various organic (suberogenic) acids [Gilson, 1890]. The relation of these acids to fatty acids is not clear, but, in view of the fact that they all lose water at higher temperatures with consequent changes such as a rise in melting point and loss of solubility in fat solvents, they would appear to be of the nature of hydroxy-fatty acids.

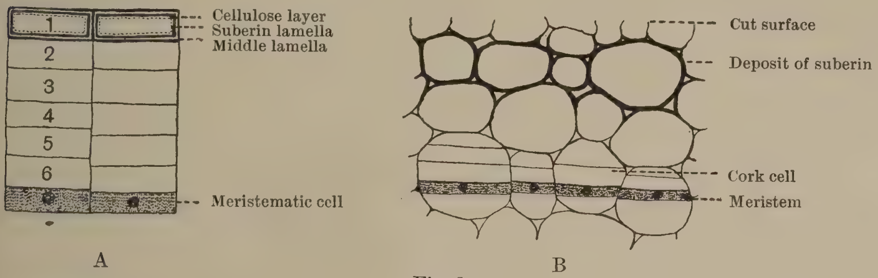

Fig. 1.

A. Series of cork cells formed from meristem, 1 oldest and outermost cell, 6 youngest. Details of wall shown in 1.

B. Formation of a new cork layer below cut surface of potato tuber.

In the present work, as a result of the experience gained with these somewhat intractable substances, the experimental methods of Gilson have been abandoned for methods similar to those used in the paint and varnish industries, where processes are involved in which linoxyn-like products are formed from the fats and oils derived from plants. It is hoped to show that such processes have considerable bearing upon the deposition of the suberin lamellae within the serried ranks of the cork cells.

EXPERIMENTAL METHOD.

The material chosen for examination was the skin of washed potatoes because it could be readily obtained in large amounts. It was washed in running water, dried fairly quickly some few feet from a fire in a well-ventilated room and then ground fairly finely.

The actual method of treatment, as now presented, is felt to be capable of yielding consistent semi-quantitative results for the various types of fatty substances involved and consists of three successive operations:

(1) Removal of chloroform-soluble material in a Soxhlet apparatus.

(2) Saponification of residual material with 3 % alcoholic sodium hydroxide.

(3) Treatment of alcoholic solution so obtained:
  (*a*) Removal of unsaponifiable material.
  (*b*) Removal of normal fatty acids.
  (*c*) Removal of hydroxy-fatty acids.

(1) *Removal of chloroform-soluble material.*

The extraction apparatus used was made of metal and had a capacity of about 2 litres; it belongs to Messrs Rowntree & Co. of York, to whom grateful acknowledgments are due. This made it possible to free from fat, in one operation, quite a large bulk of tissue. The chloroform extract was distilled to small bulk, carefully washed into a tared dish, taken nearly to dryness on a water-bath and the last traces of liquid removed by repeated evacuation—while slightly warm—in a desiccator. With samples of the same tissue, results correct to less than 1 % can easily be obtained.

(2) *Saponification with alcoholic sodium hydroxide*[1].

The extracted tissue, after removal of chloroform by exposure to air was boiled for 6 hours with about 12 times its weight of alcoholic sodium hydroxide. The alcoholic liquid was filtered off and replaced by absolute alcohol in which the tissue was boiled for 1 hour before again filtering and adding a fresh supply of alcohol. This boiling with successive lots of alcohol was continued until the filtered extract ceased to show colour. The combined alcoholic extracts were taken to dryness on the water-bath.

The residual solid matter was quite free from suberin and dissolved completely in cellulose solvents.

(3 *a*) *Removal of unsaponifiable material.*

The mass obtained after removal of alcohol was taken up in water. Usually a large volume of water was required, as some of the materials present seem to have relatively insoluble sodium and potassium soaps. The aqueous solution was repeatedly extracted with ether[2] in a separating funnel until the ethereal layer was no longer coloured and a test sample gave no stain on a watch glass. The combined extracts were concentrated, carefully washed into a tared vessel and the remaining solvent removed in a vacuum desiccator. Evacuation was repeated until constant weight was reached. The practice of repeated evacuation was adopted in preference to the standard practice for unsaponifiable fractions, namely, heating to 110° for some hours, because the presence of relatively volatile bodies was early noted and it was thought that their preservation might be useful. The solution of sodium soaps was then made acid with dilute HCl, giving a suspension of free acids.

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[1] At first it was found impossible to obtain agreement between the amounts of material in subsequent stages 3 *a*, *b* and *c*. This was found to be due in large measure to the fact that the saponifying agent was made up from rectified spirit. When it was prepared by dissolving NaOH in its own volume of distilled water and making up to the required volume with absolute alcohol, the results for subsequent stages could be consistently repeated.

[2] The ether used was purified by re-distilling after standing over stick potash for several days. Unless so treated, consistent values could not be obtained, as the ethereal extracts were found to contain varying amounts of resinous bodies.

(3 *b* and *c*) *Removal of normal acids and "hydroxy"-acids.*

It was early noted that in this mixture of acids were bodies which, although soluble in ether, could not be removed by light petroleum (B.P. 40–60°) and were similar to those described by Lewkowitsch [1909] and Fahrion [1911] and called hydroxy-acids. The method for the separation of normal and hydroxy-fatty acids described by Lewkowitsch [1909] seemed to be the only one available and proved to be suitable. The suspension was repeatedly shaken with light petroleum (B.P. 40–60°) and the solvent layer separated until a test sample gave no stain on a watch glass. The combined petroleum extracts were treated in the same manner as for chloroform-soluble fat and the amount of normal acids thus obtained.

The aqueous suspension, freed from normal acids and consisting of hydroxy-acids, was filtered and the weight of hydroxy-acids determined after drying to constant weight in a vacuum desiccator.

The filtrate freed from fatty bodies was tested for glycerol.

EXPERIMENTAL.

Sharpe's Express potatoes were used in the following experiments. It was noted that, having removed the cork layer, leaving the potato in air for a few days resulted in the formation of a second, "regenerated," cork layer, and as this involved further suberisation and might provide useful data, these regenerated layers were also taken for examination each time within one week after removal.

With samples of the same cork layer semi-quantitative numbers could be obtained. The results of duplicate experiments are given in Table I.

Potatoes of gradually increasing age up to maturity were examined, the samples being taken from the same crop at different periods. Regenerated cork layers on these potatoes were also examined. Details are given below.

Normally formed Cork Layers.

Date of collection 1924	Cork layer (code letter)	Details of potato
July 14	A	From potatoes of size slightly larger than a pea nut
	B	Larger than A. Maximum length about 1″
	C	Larger than B. Maximum length about $1\frac{3}{4}$″
August 26	D	Larger than C. Maximum length about 2″
	E	Larger than D. Maximum length about 3″
	F	Larger than E. Not quite mature
October 20	G	Larger than F. Mature

Regenerated Cork Layers.

Period of regeneration	Code letter of cork layer	Details of layer
Three weeks	H	Formed on A, B and C taken together
Three weeks	I	Formed on D
	J	Formed on E
	K	Formed on F
Four weeks	L	Formed on G

Table I.

	Weight of tissue taken g.	% on original dry weight of tissue			
		CHCl$_3$-soluble fat	unsap. material	normal acids	hydroxy-acids
Exp. 1	620	0·95	0·055	0·18	2·05
Exp. 2	663	0·95	0·058	0·17	2·17

Results of Examination of Skins A–L.

Table II. *Normally formed Cork Layers.*

	Cork layer	Dry weight % CHCl$_3$-soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxy-acids	Dry weight % normal plus hydroxy-acids
July 14	A	2·43	0·37	0·58	7·97	8·55
	B	2·74	1·86	3·12	6·28	9·40
	C	2·71	0·19	4·98	4·36	9·34
Aug. 26	D	3·30	1·53	3·23	11·23	14·46
	E	3·23	3·80	2·21	8·91	11·12
	F	3·41	4·83	5·93	6·61	12·54
Oct. 20	G	1·65	0·36	3·0	2·40	5·40

Table III. *Regenerated Cork Layers.*

Cork layer	Dry weight % CHCl$_3$-soluble fat	Dry weight % unsap. material	Dry weight % normal acids	Dry weight % hydroxyacids	Dry weight % normal plus hydroxy-acids
H	1·57	0·76	3·45	2·86	6·31
I	1·74	0·90	1·59	6·47	8·06
J	1·50	0·49	0·80	5·15	5·95
K	1·57	1·02	1·66	6·16	7·82
L	1·53	0·511	2·28	5·11	7·39

EXAMINATION OF PRODUCTS.

(1) *Chloroform-soluble fats.*

These bodies were all dark coloured, quite solid and possessed a rather sweet smell not unlike chocolate. The iodine numbers for the series A–L are given.

Table IV.

Normal cork layer		Regenerated cork	
Code letter	Iodine no.	Code letter	Iodine no.
B	70	H	74·6
C	60	I	76·0
D	68	J	74·0
F	64	K	63·0
G	56·7	L	62·4

The iodine numbers for other normal mature skins not included above, were all in the region 60–66, whilst for other regenerated cork extractions they varied from 70 to 75. Such tissues, if left in air for some weeks before extraction, yielded fats whose iodine numbers were considerably lower. A regenerated cork layer yielded a fat with an iodine number of 71, whilst after extraction 23 weeks later the iodine number was 53.

Fats from mature corky tissues had saponification values of 186–190 and 0·2–0·5 % ash in which sodium and potassium were detected. No marked solubility change was produced by exposure to sunlight for 40 days or powerful electric light for 70 days.

The amounts of constituent types of fatty acids from the fats of normal and regenerated cork were compared by saponifying them with 3 % alcoholic sodium hydroxide and following out the treatment as if for a skin.

	% unsap. material	% normal acids	% hydroxy-acids	% total acids
Fat from mature normal cork	15·3	50·0	33·6	83·6
Fat from regenerated cork	18·0	38·9	24·6	63·5

The aqueous residues after saponification were tested for glycerol, using the acrolein test. The fat from mature cork apparently contained only a trace, that from the regenerated cork evidently much more.

The normal acids thus obtained reduced alkaline permanganate slightly. They contained a little yellow colouring matter which was very difficult to remove. By repeated deposition from a mixture of light petroleum, ether and ethyl acetate, a pure white sample of an acid was obtained melting at 71–72°. Assuming mono-basicity, its molecular weight by the silver salt method was 283. The acid is assumed to be stearic acid. It had no iodine number.

The unsaponifiable material from both fats was heated to 70° on a hot plate for some hours. That from the young fat contained 23 % volatile material as against 0·2 % in the old fat. Tests of the non-volatile portions for phytosterol by heating with acetic anhydride according to usual practice were negative.

(3 *a*) *Unsaponifiable materials from cork.*

These bodies had invariably a strong penetrating odour and a yellow to brown colour. Attempts to detect phytosterol always failed. Heating to 70° removed in many cases considerable amounts of volatile material. Samples from regenerated cork contained in some cases as much as 70 % volatile bodies.

(3 *b*) *Normal acids.*

The invariable accompaniment of coloured extraneous matter, difficult to remove, has hampered progress. In order to remove it, it has been found best repeatedly to salt out the sodium salts with NaCl, finally acidifying and washing free from chloride. By this means a small amount of acid has been obtained which when moist is quite colourless but dries to a slate-grey colour. Its melting point is again 70–71°.

(3 *c*) *Hydroxy-acids.*

Although originally dark coloured, repeated salting out of the sodium salts gave a solid, quite white when moist, which again dried to a very pronounced slate colour, even *in vacuo* in the dark.

Gilson found great reduction of solubility with the suberogenic acids when heated in sealed tubes to high temperatures. Some hydroxy-acids from mature skins were heated in air for 27 days at 50°. They then showed a decrease in solubility of 50 %, indicating behaviour similar as regards solubility to the suberogenic acids. Gilson's procedure for obtaining the individual pure acids was followed without success.

DISCUSSION.

In considering the data obtained it must be remembered that the fatty products obtained are derived not only from the suberin lamella itself but also from the fatty impregnations of other lamellae. Microscopic examination has revealed in normal cork layers the presence of an occasional epidermal cell outside the cork, indicating that a trace of the original cuticle may be present. In the case of regenerated cork layers, much of the extracted material will arise from the deposit—also termed suberin—which occurs in and along the wall of cells at the exposed surface (Fig. 1 B).

The chloroform extract of normally formed cork contains very little neutral fat. The bulk appears to consist of fatty acids with only traces of sodium and potassium soaps.

The values in Tables II and III are expressed as percentages of original dry weight of tissue. Obviously, comparisons must be drawn with caution, as on different occasions different amounts of extraneous tissue may be removed with the cork and be taken for examination. On these grounds such comparisons are not made in spite of what at first sight would appear to be interesting differences in chloroform extract percentages. If the total amounts of fatty acids released on saponification be compared with the amount of chloroform-soluble material, it will be observed that the ratio

$$\frac{\text{Hydroxy-acids + normal acids}}{\text{Chloroform-sol. bodies}}$$

is approximately constant. With the exception of D, in which the yield of acids on saponification is high, the ratio for *normal cork* layers only varies from 3·3–3·67 (Table V).

Table V.

Normally formed cork		Regenerated cork	
Tissue letter	$\frac{\text{Hydroxy-acids + normal acids}}{\text{Chloroform-sol. fat}}$	Tissue letter	$\frac{\text{Hydroxy-acids + normal acids}}{\text{Chloroform-sol. fat}}$
A	3·52	H	4·02
B	3·42	I	4·6
C	3·45	J	3·96
D	4·38	K	4·9
E	3·44	L	4·8
F	3·67		
G	3·3		

When the chloroform extract is small the acids released on saponification are low also and in proportion. The modifying factor may be the amount of extraneous tissue accompanying the pure cork layers. We may accept as a first conclusion that, in the normal cork layer at all stages of development, the proportion of substance soluble in fat solvent to fatty substances extractable after saponification is approximately constant. As the series contains tissues with increasing numbers of mature cork cells, the numbers increasing from 3 or 4 to 10 or 15 without any appreciable change in the above ratio, we may conclude that there remains in the suberin lamella (Fig. 1 A) a definite proportion of material soluble in fat solvents. This result is also indicated by other investigators working on bottle cork which consists of still older cork cells, but in which Kügler [1884] found some 9 % of substances soluble in chloroform (excluding cerin). Experiments with chloroform-extracted tissues show that the chloroform-soluble material is responsible almost entirely for the staining reactions of the suberin lamella with Sudan III.

In the regenerated cork layers a larger and variable proportion of the more resistant bodies is present (Table V). Here, however, it must be remembered that we are dealing not only with internal suberin lamellae but with an irregular though continuous layer formed in and on the cellulose walls near the exposed surface, from which one would expect an excess of resistant bodies, owing to a more complete "drying" of this more exposed layer.

It can be definitely stated that the chloroform-soluble material itself contains both normal and hydroxy-acids, and Kügler's statement that stearic acid is present in a chloroform extract of *Quercus suber* adds support to the tentative identification of stearic acid in the corresponding extract of potato cork. No fatty acids have been obtained which are liquid at normal temperature, and the solid nature of all fatty bodies isolated in this work, coupled with the fact that saponification values would agree with the presence of acids of the stearic type as major constituents, suggests that only small amounts of unsaturated acids are present. The iodine value may be due to these small amounts of unsaturated acids, together with any unsaturated colouring matter or unsaponifiable body, associated with the main bulk of saturated material in the extract. It will be seen that the saponification of such an extract of normal cork yielded some 99 % of products as non-saponifiable, normal and hydroxy-fatty acids, the normal acids being mainly stearic, and showing only slight reaction to alkaline permanganate. The unsaponifiable material present in the fats is characterised by its greater content of volatile bodies in the case of regenerated cork.

After the removal of the normal and hydroxy-fatty acids from the corky tissue microscopical examination showed the complete absence of fatty materials in the residues. The walls disappeared entirely under the action of cellulose solvents, a result obtained previously by Payen [1868] with potato cork. Payen, however, before extraction with alcoholic potash had soaked the tissue for days, first in 4 % HCl and then in 20 % acetic acid. Whilst, however, the ratio of the sum of normal and hydroxy-acids to chloroform-soluble material remained constant, the amounts of each constituent were very variable (Tables II and III), this variability holding also in the regenerated cork layers. Coupling these facts, it is obvious that either the method of extraction is not reliable and the separation of hydroxy- and normal acids not efficient, or else there is genetic connection between the hydroxy- and normal acids, the one replacing the other and the whole remaining approximately constant. The consistent results in Table I negative the first alternative so that one is led to assume that the hydroxy-acids are formed at the expense of the normal acids. In the suberin lamella itself these hydroxy-acids are insoluble in fat solvents, and are only released on saponification, thus indicating some form of combination or condensation. Stearic acid is almost certainly present in the fat, and the normal acids from the products of tissue saponification seem to be very similar in all respects. The hydroxy-acids obtained by tissue saponification and by saponification of chloroform-extracted material behave similarly. These bodies plus the unsaponifiable material constitute all that is present in the walls of the cork cells. There can be little doubt that the suberin lamella owes some of its properties to the presence of normal and hydroxy-acids in a state of combination or condensation, and that the hydroxy-acids are derived from the normal acids by some process of oxidation, and if the bulk of these should consist of stearic acid, as is suspected, then perhaps saturated acids will have to be considered as a possible starting point.

Little can be said regarding the unsaponifiable material. It certainly contains no sterols. In the chloroform-extracted material glycerol is present in the normal cork in traces only but in appreciably larger amount in the regenerated cork. No glycerol has ever been detected in the alcoholic sodium hydroxide extracts. These facts are not in disagreement with the suggestion of Schmidt [1910] that the fatty acids first accumulate as glycerides, later being split up and the glycerol oxidised to CO_2 and water. The large amounts of volatile bodies present in the unsaponifiable material from regenerated cork whether from chloroform or alcoholic sodium hydroxide extracts may be by-products from the oxidative changes discussed. That these volatile bodies should be less in amount in older normal cork layers is to be expected. Dr P. W. Clutterbuck, working on the oxidation of stearic acid, has noted the appearance, as by-products, of bodies with similar appearance and properties.

SUMMARY.

A method is described by which the fatty constituents of the cork layer of the potato skin may be entirely removed, leaving the walls completely soluble in cellulose solvents. The removed bodies can be separated into chloroform-soluble material, unsaponifiable material, normal and hydroxy-acids and semi-quantitatively estimated.

It is concluded that the suberin lamella of the cork cell arises by changes

taking place in the fatty material resulting in the appearance of bodies no longer soluble in fat solvents.

A relatively constant proportion of the soluble fatty substances never undergoes this change and this soluble component of the suberin lamella is mainly responsible for its staining properties.

In the main the suberin lamella consists of relatively insoluble normal and hydroxy-fatty acid complexes which can be released by prolonged saponification as soluble soaps.

The non-saponifiable substances of the regenerated cork layers contain considerable amounts of volatile bodies.

Glycerol is found only in the chloroform extracts and then only in traces except in the case of regenerated cork layers.

The bearing of these facts on the formation of the suberin lamellae in cork cells is discussed.

The author wishes to express his most sincere thanks to Professor J. H. Priestley for constant advice and help throughout the whole of the investigation, to Mr F. C. Steward, B.Sc., who assisted in carrying out many of the extractions, to the Royal Society for a grant for collection of material, and to the Department of Scientific and Industrial Research for a grant of maintenance.

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LXIX. STUDIES ON THE GROWTH OF YEAST.

I. THE INFLUENCE OF VOLUME OF CULTURE MEDIUM EMPLOYED.

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Report to the Medical Research Council.

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Ever since the controversy between Pasteur [1872] and Liebig [1870, 1871] as to the growth of yeast in a synthetic medium the problem has remained unsettled. It will be remembered that Pasteur claimed originally [1859] that he could obtain a good growth of yeast "dans un milieu formé uniquement de sucre, d'un sel d'ammoniaque et d'éléments minéraux." Although the work of Willaman and Olsen [1923] shows that it is highly probable that the cane sugar which Pasteur used was contaminated with growth-promoting substances, yet Fulmer, Nelson and their supporters at the present time claim that they can produce a normal growth of yeast on their Medium "F" which is composed of chemicals of the highest possible degree of purity. On the other hand, there are still a number of workers who claim that for the normal growth of yeast some substance of a "bios" nature is indispensable (Funk, Eddy, Williams *inter alios*). In fact, a crystalline substance with well-defined physical properties and marked "bios" activity has recently been described by Eddy, Kerr and Williams [1924]. [For further references and details of this controversy see *Med. Res. Council Report*, 1924, p. 54.]

Whatever may be the true solution to what we may call the "bios problem," the differences between the results obtained by Pasteur and Liebig have yet to be explained on an experimental basis. The following communication is an attempt to supply such an explanation in the hope that it may throw light on the "bios problem."

Liebig, in accord with his view that fermentation was a process which only occurs in non-living matter, emphatically opposed the idea of Pasteur that it was an expression of the living yeast cell. In support of his contention he stated that he had repeatedly attempted to obtain a growth of yeast under Pasteur's conditions without succeeding in doing so. In these attempts, as we shall see below, he did not inoculate as much yeast in his medium as Pasteur had done. The main difference therefore between the experimental conditions of these two observers lay in the different volumes of medium which were employed for each yeast cell originally present. With this difference in mind, the experiments detailed in the following communication were undertaken. Their object was to determine the effects, if any, of alterations in the volume of culture medium used on the growth of single cells of yeast. The experiments fall into two classes, one in which the yeast was grown in a medium as free from "bios" as possible, the other in which various concentrations of "bios" extracts were added.

Owing to the difficulty of obtaining in any given experiment a large number of active single cell cultures, observations were also made on cultures containing two cells. The justification of the use of results obtained in two cell cultures as corroborative evidence of the behaviour of single cells will be given in the next communication (Part II).

EXPERIMENTAL.

General.

In view of the influences on growth which may occur owing to the presence of bacterial contamination (*vide infra*) the technique has been further modified to prevent contamination as far as possible and to determine with greater certainty those cases in which it may have occurred. With the exception of these modifications the general procedure was essentially the same as in the experiments previously described [Peskett, 1924]. The following media were used:

Medium 1. Basal medium + 50 g. per litre commercial cane sugar.
Medium 2. Basal medium + 50 g. per litre recrystallised cane sugar.
Medium 3. Medium 2 + 0·025 mg. per cc. of solid "bios complex."
Medium 4. Medium 2 + 1·0 cc. yeast extract per litre.
Medium 5. Medium 2 + 10·0 cc. yeast extract per litre.
Medium 6. Medium 2 + about 1·0 cc. "Kepler" malt extract per litre.

The basal medium, recrystallised cane sugar and "bios complex" were prepared as previously described. The yeast extract was prepared according to the method of Eddy, Kerr and Williams [1924] from fresh "D.C.L." yeast. After shaking with fuller's earth, the filtrate was made up to the volume of autolysate from which it had been prepared for use in these media.

It was also found to be more satisfactory to rinse the cover slips in glass-distilled water instead of acid alcohol before drying. The cultures were grown at 25°, and the yeast used was the same as in the previous experiments.

Preparation of drop-cultures.

Hanging-drop preparations were made by means of a silica pipette in the manner described previously. In view of the difficulty of removing vaseline from the cover slips before fixing and staining, this substance was only used to the extent of two small dabs placed at opposite corners of the depression in the slide. This was quite sufficient to allow the cover slip to be picked up readily with the slide. When in position, the cover slip was sealed at its edges with warm paraffin wax.

Parent culture.

As in the previous experiments, great difficulty was experienced in obtaining a large number of drops containing single cells which would grow satisfactorily. The mortality among the drop-cultures seemed to vary to a certain extent according to the manner in which the parent culture from which they were derived was obtained. After many trials it was found most satisfactory to use a culture which had been grown at 25° in 2·0 cc. of Medium 3 for not less than 2 or more than 4 days; the best results being obtained when a small loopful of a culture at least 14 days old was used for inoculation. To avoid the risk of contamination by wild yeasts, the stock culture was occasionally started afresh by transferring a colony derived from a single cell in a drop preparation to a fresh tube of Medium 3.

Drop volumes.

By direct measurement with a high power objective and a calibrated fine adjustment of the microscope the depths of a large number of different-sized drops of Medium 2 were determined. At the same time their diameters were measured by means of an eyepiece micrometer. These drops had been dusted with lycopodium powder to enable their surfaces to be focussed. Since the shape of the drops was found to be approximately that of a segment of a sphere, the usual formula was applied, viz. $V = 1/3\pi\,(3r - h)\,h^2$. From this formula the volumes of these drops were calculated and plotted on a curve against their diameters.

In the following experiments only the drop diameters were measured, and from these the volumes were read off from the curve obtained above. It will be seen therefore that the values given for drop volumes, though recorded in cubic millimetres, are only empirical.

Observation and staining of the cultures.

In order to minimise errors of evaporation and condensation of the drops, which might occur owing to changes of temperature while the cultures were under observation, a warm stage (at 25°) was used.

Fixing and staining the cultures. The technique described previously involved a long period of drying before the cultures could be fixed. Since in many cases the cultures became contaminated during this process a considerable amount of time has been spent in trying to overcome this difficulty. The technique finally adopted was as follows:

At the conclusion of the period of observation the position and approximate size of the drop-cultures were marked on the upper surface of the cover slip by means of Indian ink. The cover slip was then removed from its slide and the upper surface coated with a thin layer of paraffin wax. A small ring and an identification mark were then etched round each ink mark by means of hydrofluoric acid, the excess of acid being washed off carefully with a few drops of water. The cover slip was warmed to evaporate the greater part of the water from the drops, and whilst still warm any traces of paraffin wax or vaseline were removed with a rag moistened with xylol. The preparation was then immersed for a few seconds in a dilute solution of collodion[1], and transferred rapidly to 80 % alcohol. After a few minutes in that fluid it was transferred to 50 %, and thence to 10 % alcohol. The preparation was then stained and mounted as described in the previous experiments. The position and size of the cultures having been fairly accurately marked, it was only necessary to search these circumscribed areas for bacterial contamination.

It was found that, by using this method, only in a very few instances did any of the yeast cells of a given culture become detached from the preparation. Moreover, the final preparations were comparatively free from granular débris, even when viewed under the highest powers of the microscope, provided that the stains were filtered before use. For these reasons I have described the method at some length in the hope that it may be useful to other workers in this field.

Results.

Method of consideration.

In each experiment I have plotted on a graph the number of cells present at each observation in every culture which showed growth. All the cultures considered were found to be free from bacterial contamination according to the method described. Fig. 1 illustrates the results obtained in a typical experiment.

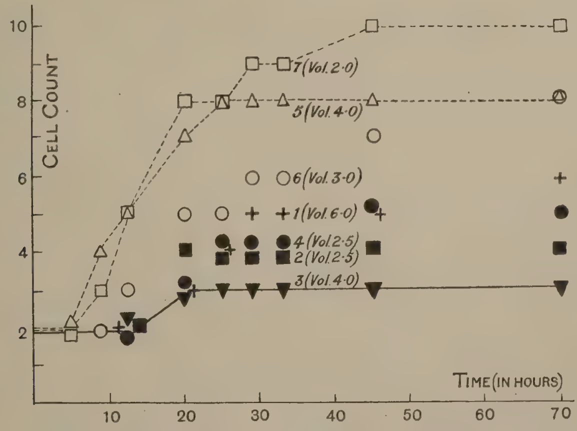

Fig. 1. Growth of two-cell cultures on synthetic medium.
Culture No. 3 (▼) showed the slowest growth.
Cultures No. 5 (△) and No. 7 (□) showed the quickest growth.

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[1] Prepared by the addition of 5·0 cc. of collodion solution (containing 3 g. of pyroxylin in 75 cc. ether and 25 cc. alcohol) to a mixture of 25 cc. alcohol and 25 cc. ether.

In order to save space, the curves have been omitted and the results given in tabular form.

Arrangement of tables.

In one experiment the points plotted on the graph fell very close together, there being little variation in the growth recorded in the individual cultures. In this experiment (4 A) the drop volumes of all the cultures are given in the first column of the table, under "Average growth."

In other experiments (1 B, 6 B, 8 A) one culture in each experiment showed considerably quicker growth on the graph, and its drop volume has been recorded in the second column of the table, under "Quickest growth." The remaining cultures in each of these experiments, showing little variation in growth, are considered in the first column of the table.

In most of the experiments the graph obtained has been similar to that shown in Fig. 1. Here it will be observed that there is considerable variation in the growth recorded in the individual cultures. In these experiments the drop volumes of the cultures showing the quickest growth (*e.g.* nos. 5 and 7 in Fig. 1) have been recorded in the second column; whilst the volumes of those showing the slowest growth (*e.g.* no. 3 in Fig. 1) have been given in the third column of the table, under "Slowest growth." The volumes of the remaining cultures in these experiments are given in the first column.

To determine the rate of growth I have in all cases studied the growth curve from the last point before the first division occurred. Any acceleration of growth due to shortening of lag period alone has not been considered.

Growth on synthetic media.

Table I. *Drop volumes in mm.*^{3}.

	A. Single cells			B. Two cells		
Exp.	Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
1	8, 58, 23, 5, 64, 90, 90, 43, 31, 14	58	10	13, 23, 64, 6, 43, 13, 14, 31	43	—
2	—	—	—	17, 10, 6, 13, 146	23	23
3	—	—	—	4, 13, 8, 5	13, 13	5
4	4, 13, 4, 13	—	—	6, 3, 2·5, 2·5	2, 4	4

In Table I the growth of 46 cultures has been considered, in volumes varying between 2 and 146 mm.^{3}. All of these were grown on "synthetic medium" (Medium 2) and found to be free from bacterial contamination so far as the technique allows it to be judged. In the range of volumes studied, there was little evidence of any constant influence of differences in volume on the growth. Thus, the evidence of acceleration in the larger volumes and retardation in the smaller shown in Exp. 3 B is counterbalanced by the uniform growth observed in the volumes shown in Exp. 4 A. In all the remaining experiments it will be seen that fairly uniform growth has been observed over a wide range of volumes; in every case in volumes larger than that in which the quickest growth has been recorded and smaller than that in which the slowest growth occurred, and *vice versâ*.

In synthetic media, therefore, within the range of volumes studied, I cannot attribute any of the variations in growth which occurred to differences in the volume of culture medium employed.

Growth on media containing "bios" extracts.

Table II. *Drop volumes in mm.³.*

		A. Single cells			B. Two cells		
Exp.	Medium	Average growth	Quickest growth	Slowest growth	Average growth	Quickest growth	Slowest growth
5	5	143, 238, 74, 90, 157, 362	90, 45	77	—	—	—
6	4	19, 26, 22, 23, 36, 48, 2, 10, 6, 13, 13, 6	115	48, 29	17, 27, 31, 17	107	—
7	6	7, 13, 9, 4	57	9, 43, 13	64, 14, 23, 48	10	1, 0·5
8	3	178, 161, 213, 260	106	—	235, 178, 178, 161, 107	270	23, 74

In Table II the growth of 57 cultures, on media containing various concentrations of growth-promoting substances, has been considered. These were all free from bacteria as far as could be judged. The volumes varied between 0·5 and 362 mm.³.

In the first place, it will be seen that in five experiments (5 A, 6 A, 7 A, 7 B, 8 B) there were cultures which showed extremes of growth in both directions. In these experiments there were 16 such cultures. As regards 15 out of these 16 it is true to say that the cultures which showed quicker growth had larger volumes than the corresponding cultures which showed slower growth and *vice versâ*. In media containing "bios" there is, therefore, evidence that the quickest growth occurs in volumes larger than those in which the slowest growth is observed.

In order to simplify the consideration of all the experiments in the table I have drawn up the following analysis. Out of 18 cultures which showed exceptionally quick or slow growth 8 showed quick growth and 10 showed slow growth.

Of the eight showing quick growth the volumes of	Exp. nos.	Of the ten showing slow growth the volumes of	Exp. nos.
Four were largest	6 A, 7 A, 6 B, 8 B	Three were smallest	7 B, 8 B
Two were smallest	5 A, 8 A	None were largest	—
Two were intermediate	5 A, 7 B	Seven were intermediate	5 A, 6 A, 7 A, 8 B

In the 9 cultures in intermediate volumes there was no constant relationship between volume and growth. Of the remaining 9 cultures, 7 showed definite evidence of acceleration of growth in the largest volumes or of retardation in the smallest, and only 2 showed the opposite effect.

Secondly then, in view of the fairly uniform growth obtained in Exp. 5 A over a very wide range of volumes, and in consideration of the aberrant growth noted in the 9 cultures in intermediate volumes (considered in the analysis) it is difficult to ascribe any of the variations in growth which occurred to differences in volume. But in those cases where extreme variations in growth occurred in association with extreme differences in volume, there was distinct evidence of acceleration of growth in the larger volumes and retardation in the smaller in the majority of cases (77 %).

Thirdly, in the media containing "bios" extracts I have not observed slowing of the growth in the largest volumes, and in only two cases out of eight did acceleration occur in the smallest volumes. Robertson [1923] claims that the growth of infusoria is approximately inversely proportional to the volume of medium in which they are grown. This is not true of the yeast used in these experiments.

Growth in presence of bacterial contamination.

Table III. *Exp.* 9. *Medium* 2. *Single and two-cell cultures.*

	After 72 hours at 25°	
Cultures considered	Grown	Not grown
16 sterile (control)	9	7
16 infected	1	15

In Table III I have recorded the results obtained in an isolated experiment in which some of the cultures were intentionally contaminated with bacteria. The infected cultures had been lightly inoculated with a pure culture of a short rod-like bacillus obtained from the laboratory air. In its cultural characteristics it resembled *B. fluorescens liquefaciens*. Although the growth recorded in this experiment was not good even in the sterile control cultures, there was a definite inhibition of growth owing to the presence of the bacteria, and a marked retardation in the single infected culture which did grow. The experiment emphasises the need for prevention of contamination by bacteria, and for means for their detection.

Mortality of the cultures.

The only experiments that I have been able to find in which the growth of isolated yeast cells has been studied in small volumes of medium are those of R. J. Williams [1919]. In these the percentage of growing cells obtained seemed to be very low, especially in his control medium, which closely resembled the synthetic medium used in my experiments. I have therefore drawn up Table IV, in which are compared the percentages of growing cells obtained in Williams' experiments and my own.

Table IV.

	A. Single cells			B. Two cells			
Medium	Cultures considered	Number growing	% growing	Cultures considered	Number growing	% growing	Observer
Synthetic	95	25	27	85	32	38	Williams
Synthetic	21	16	76	43	30	70	Peskett
"Bios"-containing	210	113	54	164	105	64	Williams
	73	37	50	34	20	59	Peskett

It will be seen that the percentage of growing cells obtained in the synthetic media was considerably greater in my experiments than in those of Williams. The percentages for the "bios"-containing media in my experiments are unduly low as an earlier experiment has been included in which the mortality was high. If this experiment is omitted the percentages are, for the single cell cultures 66 %, and for the two-cell cultures 87 %, in the media containing "bios."

The technique employed in the later experiments, *i.e.* as described in this communication, is capable therefore of yielding a high percentage of growing cells.

DISCUSSION.

The results obtained raise some points of interest.

In the first place the use of growth curves brings out clearly the variations, in some cases considerable, which may occur in the growth of individual cultures in spite of all precautions that may be taken to standardise the conditions of growth. Robertson [1923, p. 87] quotes a series of isolated observations of his cultures after a definite period of time as evidence that the growth of infusoria is approximately inversely proportional to the volume of culture medium employed. Such isolated observations appear to be of little value. For if we were to consider the growth at two definite times in the experiment shown in Fig. 1, say at 20 hours and at 70 hours, we should in the first case group together cultures 3, 1 and 4, and in the second case 5 and 6. In this way any significance of the relatively slow growth curve of culture 3 or of the relatively rapid growth of 5 would be lost. For this reason, observations of the growth have been taken at frequent intervals, and only complete growth curves have been considered in studying the effect of volume.

In the second place, it is interesting to compare the concentrations of yeast employed in these experiments with those used by Pasteur and Liebig, together with the growth obtained in each case. Pasteur inoculated "une tête d'épingle" of fresh yeast, containing 80 % water, into 100 cc. of medium and obtained a growth in one experiment amounting to 0·043 g. of yeast weighed dry. Assuming a pin's head to be approximately a sphere of 1 mm. diameter it would have a volume of about 0·5 mm.^{3}. Pasteur, then, inoculated about 0·1 mm.^{3} of actual yeast and this multiplied about 400 times.

Liebig using 100 cc. of medium inoculated with what he describes as "une trace de levûre." The only definition of this term that I can find occurs on p. 31 of his communication where he states "J'emploie de la levûre en pâte de la grandeur d'une tête d'épingle que je délaye dans 10 centimêtres cubes d'eau; je prends de celle-ci un centimêtre cube." Apart from any water that he may have used to render the yeast "en pâte" Liebig probably inoculated about one-tenth the amount of Pasteur, *i.e.* about 0·01 mm.^{3}. He failed to note any multiplication of the yeast.

If we can consider the yeast cell as a sphere of diameter about 10μ, and therefore of about 5×10^{-7} mm.^{3} volume, then the range of volumes studied in my experiments was from 4×10^6 to 3×10^8 times the volume of a yeast cell in the synthetic media, and from 1×10^6 to 7×10^8 times in the media containing "bios" extracts. Single cells in these volumes would correspond to concentrations varying from 0·025 to 0·0003 mm.^{3} of yeast per 100 cc. of medium in the former and from 0·1 to 0·00014 mm.^{3} of yeast per 100 cc. in the latter experiments. On the synthetic media the growth obtained represented a multiplication of 5 to 28 times the amount inoculated, and on the "bios"-containing media from 120 to 18,000 cells were obtained from a single cell. On medium containing commercial cane sugar (Medium 1) multiplication of about 300 to 500 times was observed over a similar range of volumes.

It will be seen therefore that growth similar to that recorded by Pasteur was only obtained in the media containing "bios," although the range of volumes in both sets of experiments included values which, for the number of cells present, were comparable with those which Liebig and Pasteur employed. In none of my experiments was there any evidence that multiplication had been less in the larger volumes. On the contrary there was some evidence to show that the quickest growth occurred in the largest volumes, in the presence of "bios" extracts. The differences between the results obtained by Liebig and Pasteur cannot therefore be ascribed to differences in the volumes of medium used per yeast cell inoculated, unless the yeast employed by them was very different from that used in my experiments. They would appear to have been due to the presence of different amounts of "bios" in the cultures.

In this connection I have observed that the appearance of the cells has been similar to that described by Pasteur ("une belle levûre très ramifiée, extrêmement jeune d'aspect, c'est à dire que les globules sont gonflés, translucides, non-granuleux") only in the media containing "bios." In the synthetic media the growth was composed of very granular cells.

By further experiments in still larger volumes, I hope to elucidate the cause of the delay in multiplication observed in large volumes by Kuster [1907] and Wildiers [1901].

Thirdly, provided that the concentration of "bios" used has not been so great as to induce the "autostatic phase" of growth, the results obtained in the media containing "bios" are difficult to bring into line with the theory of an extracellular autocatalyst for growth suggested by Robertson [1923, pp. 102 *et seq.* and p. 147]. In all the experiments in Table II the growth was of a progressively increasing character, and therefore presumably in the "autokinetic phase."

In accord with Robertson's theory we should expect to find the reproductive rate enhanced in the smaller volumes and retarded in the larger, as the cells should at the time of division lose relatively less autocatalyst in the smaller volumes than in the larger. The results given above show that any enhancement of reproductive rate that may occur cannot definitely be attributed to a smaller or a larger volume, and that on the whole the evidence points towards acceleration in the larger. In any case, Robertson's theory cannot be applied to the yeast used as no evidence of retardation of growth in larger volumes could be obtained.

Finally, the method employed includes a control of possible bacterial contamination, the importance of which has been demonstrated experimentally. Moreover, it yields a higher percentage of growing cells than that described by Williams. I hope that it may be usefully employed for the study of growth in other unicellular organisms.

SUMMARY.

1. A method is described for the study of the growth of single cells. Using this method, I have obtained a high percentage of living cells, and I have been able to detect readily those cases in which bacterial contamination has occurred.

2. The growth curves of one and two-cell cultures of yeast, grown in the absence of bacteria and in different volumes of medium, have been studied with the following results:

(*a*) In the absence of "bios." No constant effects on growth were observed as a result of differences in the volume of medium used.

(*b*) In the presence of "bios." The quickest growth occurred in larger volumes than the slowest, but there was not constant evidence to show that the growth was directly proportional to the volume. It was, however, definitely shown that the growth was not inversely proportional to the volume, as is the case in infusoria according to Robertson's observations.

3. The results summarised in 2 (*a*) and (*b*) are not in accord with Robertson's theory of an extracellular autocatalyst for growth.

4. The results obtained show that the differences between the observations of Pasteur and Liebig cannot be attributed to differences in the volumes of media employed by these workers per yeast cell inoculated, and that they were probably due to the presence of different amounts of "bios."

In conclusion I wish to express my indebtedness to Mr H. M. Carleton for his suggestion of the use of collodion in fixing the cultures and his advice throughout the elaboration of the bacteriological technique, and to Prof. Peters for his advice and interested criticism throughout the work. My thanks are also due to the Medical Research Council for a part time grant, and to the Government Grant Committee of the Royal Society for a grant to cover expenses.

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LXX. STUDIES ON THE GROWTH OF YEAST.

II. A FURTHER NOTE ON ALLELOCATALYSIS.

By GEOFFREY LEWIS PESKETT.

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Report to the Medical Research Council.

(*Received April 22nd, 1925.*)

In a recent communication Robertson [1924] has claimed that the failure of Cutler and Crump [1923] to observe allelocatalysis in cultures of *Colpidium colpoda* was due to the omission of the precaution of washing the organisms before inoculation. In my previous communication on the subject [1924] I failed to observe allelocatalysis in cultures of yeast, but I also had omitted to wash the yeast cells except in two of the experiments. In these two experiments, as stated at the time, some doubt existed as to whether the washing process had not actually added some organic matter to the culture owing to the technique employed. I have therefore repeated two typical experiments with the improved technique described in the preceding communication. Before suspending the cells in the required medium for the drop cultures, I washed them in the following manner.

METHOD.

The apparatus consisted of a large boiling tube which was plugged tightly with cotton wool at its open end. The plug was covered with fine-meshed muslin to prevent pieces of it from becoming detached and drawn down into the medium during the centrifugalisation. The edges of the muslin were firmly attached to the rim of the tube to keep the plug in position. Through the plug and muslin passed a Dreyer's pipette also plugged at its wider end. In the boiling tube were placed five small test tubes of narrow bore, separated from the bottom of the tube by a small pad of cotton wool. These tubes bore permanent identification marks so that they could be readily distinguished from each other. Before filling them with medium the whole apparatus was thoroughly cleansed and sterilised. From 2·0 to 2·5 cc. of the sterile synthetic medium (Medium 2) were then placed in each of the tubes under aseptic conditions, and the whole was again sterilised by steam heat in the Koch steriliser for 45 minutes on three successive days.

About 0·25 cc. of the culture containing the cells to be washed was admitted to the apparatus by a capillary pipette inserted into the Dreyer pipette. The plug was replaced with the usual precautions and a rubber teat was used to force the fluid containing the cells down into the first tube of synthetic medium, which was then gently agitated by passing a few bubbles of air through it. After standing a few minutes the whole apparatus was placed in a large centrifuge. The cells, which in the case of the yeast used sink very readily, were brought to the bottom by 30 to 45 seconds' centrifugalisation at about 600 r.p.m. The supernatant fluid was now pipetted off and a fresh amount of medium introduced from the next tube. The washing and centrifuging were then repeated.

It was found that the cells could be very thoroughly washed by this method with only the slightest risk of infection, provided that the apparatus was so arranged and care was taken to see that the Dreyer pipette was only moved in the wool plug as little as possible, in both horizontal and vertical directions. Moreover, the mortality among the washed cells was 50 % in Medium 1 and 28 % in Medium 2—figures which compare favourably with those for the previous experiments in which the cells were not washed.

RESULTS.

By the technique described above, the cells used in these experiments were washed five times with from 2·0–2·5 cc. of synthetic medium. Assuming that one-tenth of a cc. of medium was left after each washing, then the concentration of any extraneous matter introduced with the cells in the first instance was reduced after washing five times to less than 1/3,000,000. At the end of the washing process we can assume, therefore, that we are dealing with yeast cells in synthetic medium free from any impurities other than those which may be present in the medium itself owing to the difficulty of preparing absolutely pure chemicals.

After washing, some of the cells were suspended in synthetic medium (Medium 2), others in the medium containing commercial cane sugar (Medium 1). The results are given in the following table in which is recorded the number of cells present after a given time in cultures which contained originally one, two or three cells. The cultures were grown at 25° and observations taken as described in Part I.

Exp. 10, Medium 1. Growth after 34 hours at 25°.

1 cell	2 cells
10, 8	9, 12, 9, 19

The corresponding drop volumes were, in mm.^{3},

103, 23	23, 16, 16, 16

Exp. 11. Medium 2. Growth after 45 hours at 25°.

1 cell	2 cells	3 cells
3, 5, 4, 2	10, 7, 8, 3, 5, 4, 5	9, 6

The corresponding drop volumes were, in mm.^{3},

106, 23, 106, 19	10, 17, 23, 19, 17, 17, 51	31, 12

No cultures in these experiments have been excluded from the table on account of exceptionally high growth, and all were examined after fixing and staining and found to be free from bacteria. The absence of any effect which could be termed allelocatalytic was confirmed at all the other periods at which the cultures were examined, as well as at the times recorded in the table. The results show that allelocatalysis does not occur in the case of the yeast studied even after washing the cells before inoculation.

Conclusion.

The failure to observe allelocatalysis in the experiments recorded previously was not due to the omission of the precaution of washing the cells before inoculation. My previous conclusion was therefore justified, namely, that allelocatalysis does not occur in the case of the yeast studied.

My thanks are due to the Medical Research Council for a part time grant, and to the Government Grant Committee of the Royal Society for a grant to cover expenses.

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LXXI. FERMENTATION BY DRIED YEAST PREPARATIONS.

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(*Received April 23rd, 1925.*)

Some years ago the behaviour of zymin (yeast dehydrated by acetone) with different volumes of sugar solution was described by Harden and Young [Harden, 1911, p. 37].

When zymin is incubated with 10 cc. of approximately 10 % glucose solution at 25°, fermentation commences almost immediately, increases to a maximum rate in about 40 minutes and then very slowly decreases in rate. If, however, 25 cc. of the glucose solution of the same concentration be employed with the same weight of zymin, only a very slow fermentation occurs for the first hour and a half, after which the rate increases and finally becomes only slightly less than that in the presence of 10 cc. of the glucose solution. The curves *A* and *B* in Fig. 1 show this behaviour in typical manner, but the actual volume of gas evolved varies with different samples of zymin. A similar phenomenon is shown by dried yeast (Fig. 2) but the difference in behaviour is often still more marked. When larger volumes of glucose solution are taken the onset of fermentation is still further delayed or may even be entirely prevented. This is shown by the following results:

A. 1 g. zymin + 5 cc. of 10 % glucose solution + 0·2 cc. toluene.

B. 1 g. zymin + 20 cc. of 10 % glucose solution + 0·2 cc. toluene.

Fermentation commenced at once in A, at the rate of about 0·6 cc. per 5 minutes, and at the end of 7 hours 66 cc. of CO_2 had been evolved, whereas no fermentation was observed in B, even after 23 hours.

The autofermentation of zymin is affected in a similar manner. Thus 2 g. zymin (1) + 10 cc. H_2O, and (2) + 25 cc. H_2O at 25° gave the results shown in curves *A* and *B*, Fig. 3.

These observations have evidently an important bearing on those discussed by Abderhalden and Fodor [1921] and subsequently studied by Sobotka [1924].

The thesis maintained by Abderhalden and Fodor is that the complex chemical action of yeast on sugar is only reproduced by preparations such as acetone-yeast (zymin), yeast-juice, maceration extract or dried yeast, in so far as the original colloidal condition of the protoplasm of the yeast cell is retained. Without at the moment discussing this interesting and important conclusion, for and against which much can be said, attention may be directed to one of the arguments adduced which appears to be founded on a misunder-

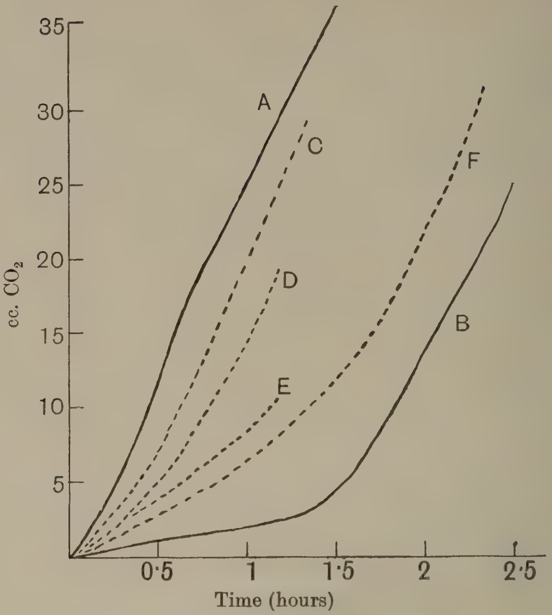

Fig. 1. 2 g. zymin in each + 0·2 cc. toluene.

A. 10 cc. of 10 % glucose } Solutions added direct.
B. 25 cc. „ }
C. 25 cc. „ ; 0·2 *M* $NaC_2H_3O_2$ } Solutions added after preliminary moistening for 1 minute.
D. 25 cc. „ ; 0·1 *M* „ }
E. 25 cc. „ ; 0·05 *M* „ }
F. 25 cc. „

(The curve for 0·025 *M* acetate is identical with *E*.)

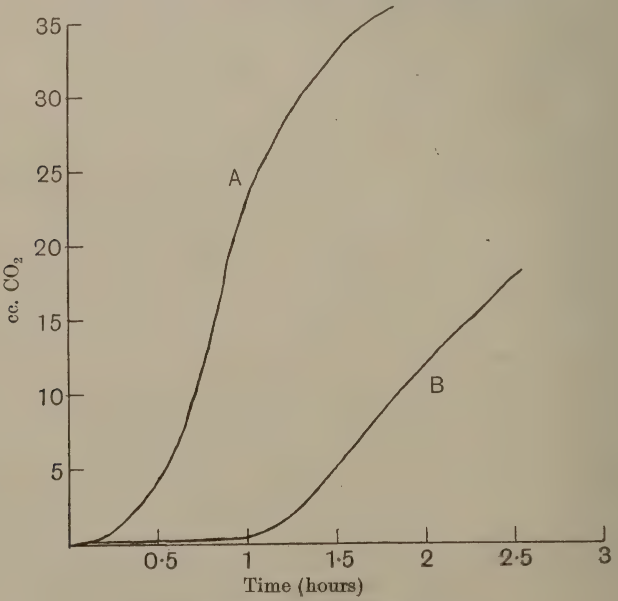

Fig. 2. 2 g. dried yeast + 0·2 cc. toluene.

A. + 10 cc. of 10 % glucose } added direct.
B. + 25 cc. „ }

standing. One of the marked differences between living yeast and yeast preparations is alleged to be that these preparations are incapable of fermenting dilute sugar solutions (p. 143). "Ebensowenig wie Hefeauszüge vermag sterile Trockenhefe (Acetondauerpräparat oder durch Alter sterilisierte Hefe) verdünnte Zuckerlösungen zu vergären, weil allen diesen Stoffen die Fähigkeit der Konzentration, die nur lebenden Zellen eigen ist, fehlt." The statement appears to be founded on the fact that Buchner [1903, p. 260] used 40 % cane sugar in his fermentation experiments with zymin, and only in one instance published results obtained with another concentration. Buchner, moreover, used 4 g. cane sugar and 10 cc. of water to 2 g. zymin (Dauerhefe), a proportion which gives almost immediate fermentation.

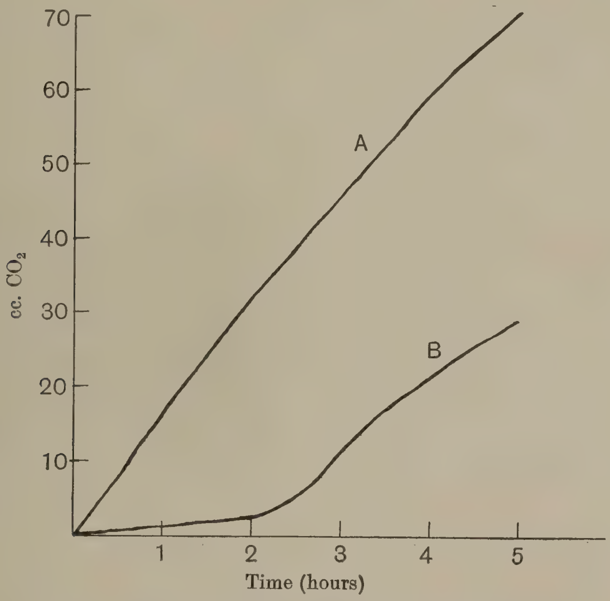

Fig. 3. Autofermentation of 2 g. zymin + 0·2 cc. toluene.
A. + 10 cc. H_2O } added direct.
B. + 25 cc. H_2O }

Actually, more dilute solutions of glucose are fermented by zymin more rapidly than 40 % solutions, provided that only a small volume of the solution is used.

In Table I are given figures for 10 cc. and 25 cc. respectively of each of the concentrations 2·5, 5, 10, 20 and 40 g. of glucose per 100 cc. with 2 g. zymin.

Abderhalden and Fodor made experiments using 10 g. dried yeast, 10 g. sugar and 250 cc. of H_2O and it is therefore not a matter for surprise that they obtained long induction periods, followed by fermentation due to the growth of yeast cells.

Table I.

2 g. zymin with (*a*) 10 cc. glucose solution + 0·2 cc. toluene } at 25°.
(*b*) 25 cc. glucose solution + 0·2 cc. toluene }

cc. of CO_2 evolved.

Time of incubation (mins.)	(*a*) 10 cc. Concentration, g. per 100 cc.					Time of incubation (hrs.)	(*b*) 25 cc. Concentration, g. per 100 cc.				
	2·5	5	10	20	40		2·5	5	10	20	40
10	1·8	3·3	2·1	2·5	2·5	1	2·7	2·2	3·6	3·6	4·5
20	4·4	8·1	5·1	5·2	4·5	2	3·0	2·7	7·8	7·7	6·4
30	8·4	12·5	8·7	7·9	5·9	3	15·0	11·1	17·8	16·2	8·7
40	12·8	16·0	11·6	10·6	7·2	8	71·4	58·1	64·5	47·8	30·0
50	16·5	20·0	14·0	13·7	8·4						
60	19·9	24·6	16·5	15·9	9·9						
130	43·8	50·1	38·1	32·8	19·8						

Sobotka, who has more recently taken up the study of fermentation by dried yeast [1924] has carried out further work on the same lines as Abderhalden and Fodor and has come to the conclusion (which he also ascribes to them, erroneously as it seems to me) that the effect produced by dried yeast is to be attributed exclusively to living cells; "die Leistung der Trockenhefe ausschliesslich auf lebende Zellen zurückzuführen ist" (p. 9).

Sobotka used 1 g. of dried yeast with 1 g. glucose and 20 cc. of water and observed periods of induction of 80–260 minutes with one group of yeasts and 4–19 hours with another group. Only one experiment on the effect of yeast concentration is recorded. In this the quantity of yeast per 20 cc. of 5 % glucose was varied and the resulting very marked decrease in the incubation period explained as being due to the shorter time required for the production of the necessary number of cells capable of fermenting.

There seems to be little doubt that Sobotka's results were actually due to the growth of living yeast cells contained in his preparations but his general conclusion is too sweeping. The "Dauerhefe" prepared by Buchner and Albert was carefully tested by them and found to be free from yeast cells capable of growth and yet they attained good and immediate fermentation by its use. Three successive samples of zymin prepared in this laboratory were also found to be free from yeast cells capable of growth, so that the undoubted fermentative powers of these samples must be attributed to the enzyme contained in the dead cells. Now ordinary dried yeast, which admittedly contains living yeast cells, behaves in an analogous manner to zymin when brought into different volumes of sugar solution and it is therefore only reasonable to attribute part at least of the fermentation produced by dried yeast to the enzyme in the dead cells.

No entirely satisfactory explanation of the phenomenon has as yet been found. The facts suggest that the problem, as suggested by Abderhalden and Fodor [1921], is concerned with the dispersion of the enzyme. If we suppose that this occurs rapidly in a more concentrated solution of the extractives of zymin, less rapidly in a more dilute solution, the long induction period in a large volume of sugar solution would be explained. In favour of this view is the fact that the onset of fermentation can be greatly hastened in two different ways, each of which is consistent with the foregoing suggestion, viz. by

(1) The addition of a boiled extract of the dried yeast.

(2) The addition of certain salts, *e.g.* sodium acetate or sodium bicarbonate.

On the other hand the action of these salts is to some extent specific and experiments still in progress indicate that washing zymin with sodium acetate solution does not remove the enzyme any more rapidly than does washing with distilled water, as would be expected if it caused rapid dispersion of the enzyme. Various other possible explanations suggest themselves and these are now being investigated.

The effect of an extract of the dried yeast and of sodium acetate or bicarbonate solution is exhibited in the following experiments.

A. *Extract of zymin or of dried yeast.*

(1) *Zymin.*

2 g. of zymin + 0·2 cc. toluene were treated at 25° in each case with (1) 1 g. glucose + 10 cc. H_2O; (2) 1 g. glucose + 10 cc. of an extract of 4 g. zymin in 20 cc. of boiling water; (3) 2·5 g. glucose + 25 cc. H_2O; (4) 2·5 g. glucose + 25 cc. of an extract of 4 g. zymin in 50 cc. boiling water. The resulting solutions of yeast extractives in (2) and (4) were therefore approximately twice the concentrations of those in (1) and (3). As seen from the figures showing the course of fermentation, the addition of extract hastened the onset of fermentation slightly in the case of 10 cc., very considerably in that of 25 cc. and further, as would be expected, increased the rate of fermentation owing to the additional phosphate and co-enzyme added.

	cc. CO_2 evolved.			
	2 g. zymin + 1 g. glucose		2 g. zymin + 2·5 g. glucose	
Time (hours)	10 cc. H_2O	10 cc. extract	25 cc. H_2O	25 cc. extract
0·25	1·4	7·7	0	2·8
0·5	5·6	17·6	0	5·2
1·0	16·5	31·8	1·5	11·2
2·0	36·4	52·5	10·5	23·9
3·0	—	—	28·8	46·8

(2) *Dried yeast.*

(*a*) 2 g. dried yeast + 2·5 g. glucose + 0·2 cc. toluene in each case: (1) + 25 cc. H_2O; (2) + 25 cc. extract made by heating 4 g. dried yeast with 50 cc. H_2O in a boiling water-bath for 17 minutes. No fermentation was produced in 20 hours in the absence of extract; whereas in its presence fermentation commenced after an hour and a half.

(*b*) 2 g. dried yeast + 1 g. glucose + 0·2 cc. toluene in each case: (1) + 10 cc. H_2O; (2) + 10 cc. extract made by heating 4 g. of dried yeast with 20 cc. H_2O in a boiling water-bath.

Time (hours)	cc. CO_2 evolved. 2. g dried yeast + 2·5 g. glucose 25 cc. H_2O	 25 cc. extract	2 g. dried yeast + 1 g. glucose 10 cc. H_2O	 10 cc. extract
0·25	0	0·2	0	1·3
0·5	0	0·7	0·2	3·2
1·0	0	1·5	0·7	8·6
1·5	0	3·6	3·8	16·2
2·0	0	6·9	10·2	23·2
3·0	0	17·0		

B. *Sodium acetate.*

Preliminary experiments showed that the substitution of 0·2 *M* $NaC_2H_3O_2$ for water caused a great diminution of the "period of induction" without serious change of p_H and further investigation showed that the effect was a maximum at about 0·2–0·3 *M* concentration of the acetate. No analogous effect could be produced with sodium chloride. Some irregularity was at first obtained in the results with different concentrations, but it was found that this could be obviated by adding to the 2 g. of zymin 5 cc. of water, shaking for 1 minute and then adding 15 cc. of a solution containing 2·5 g. of glucose and 5 cc. of acetate of the requisite concentration.

Typical fermentation curves for zymin with a series of concentrations of $NaC_2H_3O_2$ are given in Fig. 1, *C*, *D*, *E*, *F*. Similar results were obtained with dried yeast.

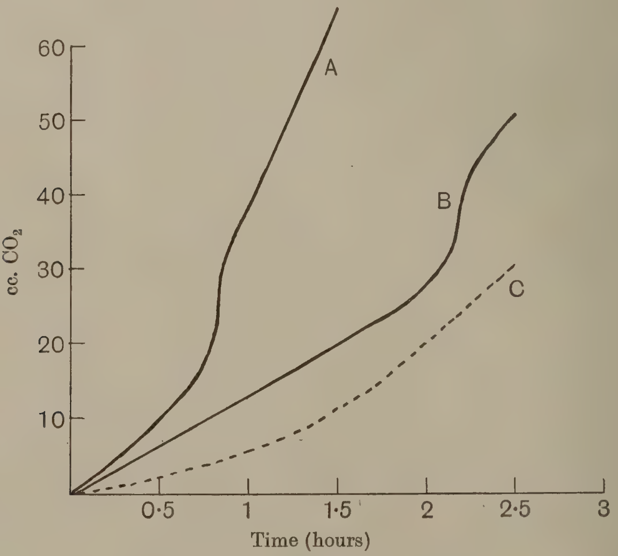

Fig. 4. 2 g. zymin + 0·2 cc. toluene.

A. +25 cc. of 10 % glucose; 0·05 *M* $NaHCO_3$
B. " " ; 0·1 *M* $NaHCO_3$
C. " "
} Solutions added after preliminary moistening for 1 minute.

C. *Sodium bicarbonate.*

Sodium bicarbonate produces a similar effect, the most favourable concentration, under the conditions employed above, being 0·1 *M*. Regular results were only obtained by adopting the same method of adding the solutions as was used for the acetate (Curves *A* and *B*, Fig. 4). An interesting point of difference between the curves obtained with acetate and bicarbonate is that the latter show a pronounced "phosphate effect" whereas the former do not.

SUMMARY.

1. Air-dried yeast and zymin (yeast dehydrated by acetone) produce fermentation rapidly in a small volume of sugar solution, only after considerable delay in a large volume.

2. The addition of yeast extract or solutions of certain salts such as acetates or bicarbonates greatly accelerates the onset of fermentation in presence of a large volume of sugar solution.

3. Air-dried yeast possesses a definite fermenting power which is not dependent on the presence of living cells.

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LXXII. THE ESTIMATION OF PHOSPHORUS AND MAGNESIUM.

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I. Phosphorus.

In the estimation of phosphorus, the highest accuracy, undoubtedly, is to be attained by the use, in conjunction with a delicate micro-balance, of some gravimetric method in which the phosphorus is precipitated as a compound of which it forms only a small percentage. Gravimetric methods, however, are apt to be long and troublesome. Particularly is this the case when the amount of substance to be estimated is very small, so that slightly incomplete drying of the precipitate, for example, may introduce a very considerable percentage error into the final result. Although the colorimetric method described by Bell and Doisy [1920]—especially in the modified form introduced by Briggs [1922]—is rapid and convenient, we have had difficulty in obtaining uniformly accurate results by its use, except in the case of inorganic phosphorus. The main sources of error in the estimation of acid-soluble and total phosphorus have been pointed out by Martland and Robison [1924]. They reduce the amount of sulphuric acid used in the ignition since they find that the amount used by Briggs, with that added in the molybdate solution, may bring the total acidity near, or even above, the limit of safety. Further, they allow the ignition to continue for as short a time as possible, to prevent loss of phosphorus by absorption by the glass.

Another source of error, which applies to estimations of inorganic phosphorus, is introduced by the trichloroacetic acid, which these authors find to contain an impurity, which itself gives a blue colour with the Briggs reagent. This error is serious only when the colorimetric readings are made after long standing, and the interfering substance may be removed by distilling the trichloroacetic acid. Although, by incorporating these modifications in the method, accuracy may be attained, we desired to avoid, if possible, the use of colorimetry, and therefore turned our attention to volumetric methods.

It has long been customary, in determining the phosphorus content of steel, etc., to precipitate the phosphorus as ammonium phosphomolybdate, to dissolve the precipitate in standard sodium hydroxide solution, and to titrate the excess alkali with standard acid. This method, which was originated by Pemberton [1893], is very well known. The amounts of phosphorus dealt with, however, have been much greater than those present in the small volume of blood available for analysis, and it was therefore our first task to see whether the method was sufficiently delicate to estimate with reasonable accuracy the 0·05 mg. of inorganic phosphorus present in 2 cc. of normal blood. This, once the appropriate conditions of precipitation and titration had been determined, was found to be the case; it was possible to estimate, within 3 %, 0·05 mg. of phosphorus contained in 20 cc. of fluid. A second series of experiments showed that exactly the same conditions with respect to quantity of precipitant, etc., sufficed for the estimation of six times that concentration of phosphorus with, indeed, somewhat greater accuracy. These results (Table I) showed that the method was capable of estimating the inorganic phosphorus in 2 cc. of blood through a range extending from below to far above the normal value. For total, or acid-soluble phosphorus, even less blood would be required.

Table I. *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Weight of phosphorus in mg.		Error
Present	Found	%
0·05	0·0514	3
	0·0485	3
	0·0505	1
	0·0497	0·6
0·10	0·0999	—
	0·101	1
	0·101	1
	0·0995	0·5
0·15	0·150	—
	0·152	1
0·20	0·198	1
	0·199	0·5
	0·199	0·5
	0·201	0·5
0·30	0·298	0·6
	0·305	1·6
	0·302	0·6
	0·297	1

There remained a further important point to be determined. Bell and Doisy [1920] have pointed out that in their blood filtrate the inorganic phosphate increases on standing, becoming, after the lapse of a few hours, equal to the acid-soluble phosphorus, owing to the gradual hydrolysis in acid solution of the organic phosphorus-containing substances present. Martland and Robison [1924], on the other hand, have carried out experiments which appear to show that the hydrolysis is enzymic and practically ceases after the addition of trichloroacetic acid. Hence they recommend that the laking of the blood and the precipitation of proteins by trichloroacetic acid (1 %) be carried out as quickly as possible, whilst Bell and Doisy consider it essential that the whole estimation be carried out at once. In face of these contradictory results, we considered it necessary to determine with what rapidity the estimation must be carried out. For our experiments on the point to have any force, however, the precipitation of ammonium phosphomolybdate must be complete in a few minutes so that the estimation may be carried out rapidly; and equally, on longer standing, there must be no further precipitation—*e.g.* of ammonium tetramolybdate or of molybdic acid. That this condition is amply satisfied is shown by the results recorded in Table II, from which it appears that the precipitation of phosphorus from a solution of potassium phosphate is complete in 10 minutes, and is not increased when the reaction mixture is allowed to stand for as long as 14 hours. The second part of Table II gives the results of our experiments on the time factor in the case of blood. It will be seen that they confirm the results of Martland and Robison [1924], showing that, whilst it is necessary to precipitate the blood proteins as rapidly as possible after laking, the amount of inorganic phosphate in the protein-free acid filtrate undergoes little or no change on standing.

Table II.

(*a*) *Estimation of phosphorus in a solution of pure potassium dihydrogen phosphate.*

Time allowed for precipitation	Weight of phosphorus in mg.	
	Present	Found
5 mins.	0·20	0·12
10 ,,	0·20	0·199
15 ,,	0·20	0·198
	0·20	0·201
20 ,,	0·20	0·201
	0·20	0·201
30 ,,	0·20	0·199
60 ,,	0·20	0·201
14 hours	0·20	0·201
	0·20	0·199

(*b*) *Estimation of inorganic phosphorus in blood.*

		mg. P/100 cc.
I.	Blood laked and proteins precipitated at once Inorganic phosphorus estimated in filtrate at once. 15 mins. allowed for precipitation of ammonium phosphomolybdate	3·86
	Inorganic phosphorus estimated at once. 2 hours allowed for precipitation of ammonium phosphomolybdate	3·86
	Inorganic phosphorus estimated after allowing protein-free filtrate to stand 2 hours at room temperature. 15 mins. for precipitation of ammonium phosphomolybdate	3·90
II.	Blood laked and allowed to stand 1 hour at room temperature. Proteins then precipitated and inorganic phosphorus estimated in filtrate at once. 15 mins. for precipitation of ammonium phosphomolybdate	4·60

Still further preliminary experiments with known amounts of phosphorus showed that the accuracy of the method was not affected by the presence of such substances as are either found normally in blood and urine or are added in preparing them for analysis. There was no loss of phosphorus during ignition with sulphuric and nitric acids for the removal of organic matter (Table III).

Table III.

(*a*) *Potassium dihydrogen phosphate added to urine. Inorganic phosphorus estimated.*

Weight of phosphorus added in mg./100 cc.	Weight of phosphorus found in mg./100 cc.	% of added phosphorus recovered
—	63·0	—
32·2	95·8	101·8
61·7	124·2	99·2
105·5	168·0	99·5

(*b*) *Salts etc. added to a solution of pure potassium dihydrogen phosphate.*

	Weight of phosphorus in mg.	
Substance added	Present	Found
Sodium sulphate (2·5 g.)	0·10	0·099
Trichloroacetic acid (0·4 g.)	0·20	0·199

(*c*) *Solution of pure potassium dihydrogen phosphate, with added cane sugar ignited with the sulphuric-nitric acid mixture.*

Weight of phosphorus in mg.	
Present	Found
0·10	0·101
0·20	0·199

Method.

20 cc. of the liquid to be examined were measured into a 100 cc. beaker, and 1 cc. concentrated nitric acid and 3 cc. 32 % ammonium nitrate were added. The beaker was heated to 70–75° on the water-bath, and, after the addition of 5 cc. 3·5 % ammonium molybdate, was re-heated to that temperature. It was then allowed to stand and cool. After not less than 10 minutes —we adopted 15 as the standard time—the liquid was filtered through paper pulp which should be free from scraps of unpulped paper. We have found that the presence of such scraps hinders very considerably the complete solution of the precipitate in the standard alkali. In the filtration, only a moderate suction should be used. The precipitate was then washed, first with a 2 % solution of nitric acid, and finally with a 2 % solution of potassium nitrate until the washings were free from acid. Usually, two washings with acid and four with potassium nitrate were sufficient, using 20 cc. for each washing. The paper pulp and precipitate were then returned to the beaker, the funnel and glass rod being washed with the potassium nitrate solution, and not with distilled water, which decomposed the precipitate giving high values. The precipitate was then dissolved in a known volume of standard sodium hydroxide, and the excess alkali determined by titration with standard acid.

The indicator used in the titration was phenolphthalein, and it was therefore necessary to use CO_2-free alkali. Moreover, since shaking to dissolve the precipitate in the open air inevitably caused the absorption of an appreciable —and varying—amount of CO_2, it was necessary to carry out the titration in a CO_2-free atmosphere. The acid and alkali (CO_2-free) were kept in paraffined bottles from which they were siphoned into burettes by means of the arrangement described by Cole [1920]. The delivery tubes of the burettes passed through holes in a rubber stopper which fitted the mouth of the beaker used in the precipitation. Through this stopper passed also a third, small, burette, from which the indicator was added, and two glass tubes by means of which a stream of air was drawn through the beaker during the titration. Carbon dioxide was removed from the air by means of soda-lime. The air inlet tube reached almost to the bottom of the beaker so that the air current not only removed CO_2 from the potassium nitrate solution used to wash in the precipitate, but kept the liquid well stirred. The beaker containing the precipitate was placed in position and the air current was started. Two minutes sufficed to remove CO_2 from the potassium nitrate solution and from the air in the beaker. 10 cc. $N/70$ sodium hydroxide were then added from the (20 cc. capacity) burette. The precipitate dissolved readily unless the quantity of ammonium phosphomolybdate was too great, in which case a second 10 cc. were added. As soon as the precipitate was completely dissolved, a drop of phenolphthalein was added, and the excess alkali was titrated with $N/70$ sulphuric acid.

1 cc. $N/70$ sodium hydroxide used in dissolving the ammonium phosphomolybdate was equivalent to 0·0198 mg. phosphorus. The factors given in the literature vary enormously, for though the precipitate is here referred to as ammonium phosphomolybdate its composition is not by any means constant but appears to depend on the conditions under which it is formed. Whatever its actual composition may be, however, a large number of determinations has shown that it does not vary when the reasonably elastic conditions we prescribe are adhered to. The factor given above is based on our own work.

By the use of still more dilute standard solutions it would, no doubt, be possible to achieve greater accuracy in dealing with the very small amounts of inorganic phosphorus met with in normal blood, but we chose $N/70$ solutions for two reasons. Firstly, they cover adequately the required range, since with 0·05 mg. phosphorus the maximum error is only 3 % and the accuracy increases with increasing amounts of phosphorus up to 0·30 mg. Secondly, we employ solutions of this strength for other routine purposes such as the estimation of non-protein nitrogen and urea in blood.

Since the work described in this paper was completed, our attention has been called to a somewhat similar method for the estimation of phosphorus in urine and faeces [Richards and Godden, 1924]. These authors, however, deal with amounts of phosphorus vastly greater than those available in blood analysis. To overcome the difficulty of absorption of CO_2 by the alkali, they dissolve the precipitated ammonium phosphomolybdate in standard alkali (0·5 N), boil, add a known volume of standard acid in excess of that required for neutralisation and boil to remove CO_2. After cooling, the solution is titrated with standard alkali. Not only is this procedure longer than our method of titration in a CO_2-free atmosphere, but it does not appear to us to be equally satisfactory for work on the micro scale, since it does not give so rigorous an exclusion of carbon dioxide.

Preliminary treatment of blood and urine.

Urine. 5 cc. urine were diluted to 250 cc. with distilled water. For *inorganic phosphorus*, 20 cc. of this solution were used and precipitated direct. For *total phosphorus*, 20 cc. were evaporated to small bulk in a wide boiling tube. 2 cc. of a mixture of three parts of concentrated nitric acid and one part of concentrated sulphuric acid were then added, and the mixture heated until nitrous fumes ceased to be evolved and white fumes of sulphur trioxide were just beginning to appear. When necessary, more nitric acid was added and heating continued in the same way until, after removal of the nitric acid, the residual solution was colourless. This was diluted with about 5 cc. distilled water and neutralised (to methyl orange) with concentrated sodium hydroxide solution. The neutral solution was then transferred quantitatively to a 100 cc. beaker for precipitation. The total volume with the washings was about 25 cc. *Organic phosphorus* was estimated by difference, but where the increased accuracy of a direct determination is required, the procedure of Taylor and Miller [1914] may be carried out. To 20 cc. urine in a 25 cc. flask they add powdered barium hydroxide until the liquid is just alkaline, make up the volume to 25 cc. with water and filter. To 20 cc. of the filtrate in another 25 cc. flask, they add dilute sulphuric acid until the liquid is slightly acid, fill up to the mark with water, and filter from the precipitated barium sulphate. 1 cc. of this second filtrate is equivalent to 0·64 cc. of the original urine. For the volumetric determination of organic phosphorus it should be diluted and treated as described above for total phosphorus. The determination of inorganic or organic phosphorus should be carried out on fresh urine.

Blood. For the determination of *total phosphorus* in blood, 0·5 cc. was treated with 2 cc. of the sulphuric-nitric acid mixture as described for urine. For *inorganic* and *acid-soluble phosphorus* 5 cc. blood were laked as quickly as possible by adding to 30 cc. distilled water in a 50 cc. flask. 5 cc. of a 25 % solution of trichloroacetic acid were added at once, and the liquid was diluted to 50 cc. After standing for 5 minutes it was filtered. 20 cc. of the filtrate (equivalent to 2 cc. blood) were used for the direct precipitation of the inorganic phosphorus; 10 cc. were treated as described for total phosphorus in urine for the determination of the total acid-soluble phosphorus.

A few typical results with blood and urine are given in Table IV. The colorimetric method used for comparative purposes was that of Briggs [1922] as modified by Martland and Robison [1924].

Table IV.

Phosphorus; mg./100 cc.

Inorganic		Acid soluble		Total	
Colorimetric	Titrometric	Colorimetric	Titrometric	Colorimetric	Titrometric
(1) *Blood.*					
3·75	3·76	26·60	26·40	34·4	34·8
3·40	3·33	23·64	23·59	—	—
4·40	4·43	22·20	22·10	—	—
3·28	3·23	25·20	25·00	—	—
2·96	2·99	22·20	22·08	—	—
3·35	3·34	23·40	22·97	—	—
3·28	3·31	26·67	26·83	35·8	35·5
(2) *Urine.*					
69·65	69·53	—	—	72·36	72·20
41·66	41·85	—	—	43·55	43·68

II. MAGNESIUM.

Many of the methods at present in use for the estimation of magnesium in blood and serum depend on the estimation—usually colorimetric—of the phosphorus content of ammonium magnesium phosphate. It is therefore obvious that our method for the determination of phosphorus affords a means of estimating magnesium titrometrically. The average normal magnesium content of blood serum is 2·5 mg. per 100 cc.; 2 cc. serum thus contain 0·05 mg. magnesium, which corresponds to 0·129 mg. phosphorus, and is therefore well within the range of our method.

In dealing with serum, calcium is first precipitated as described by Kramer and Tisdall [1921]. 2 cc. serum are added to 2 cc. water in a 15 cc. centrifuge tube; 1 cc. of a saturated solution of ammonium oxalate is added, and the volume made up to 6 cc. with distilled water. After standing for half an hour, the precipitated calcium oxalate is removed by centrifuging. From 5 cc. of the supernatant liquor, magnesium is precipitated by the method of these authors. 1 cc. of a solution of ammonium phosphate and 2 cc. concentrated ammonia (0·880) are added and the mixture is allowed to stand overnight. (The solution of ammonium phosphate is prepared by dissolving 25 g. di-ammonium hydrogen phosphate in 250 cc. water, adding 25 cc. concentrated ammonia, allowing to stand overnight, filtering, boiling off excess ammonia, and diluting to 250 cc. For use, 20 cc. of this solution are diluted to 100 cc.) Next morning, the liquid is centrifuged and the precipitate washed by centrifuging three times with 5 cc. ammonia solution (10 cc. concentrated ammonia in 100 cc.). The precipitate is then dissolved in 5 cc. 2 % nitric acid; the solution is transferred quantitatively to a 100 cc. beaker, and the phosphorus estimated as usual. 1 cc. $N/70$ sodium hydroxide is equivalent to 0·0151 mg. magnesium. Table V gives a few results from standard solutions of magnesium sulphate and from blood serum.

Table V. *Estimation of magnesium.*

(1) In a solution of pure magnesium sulphate

mg. magnesium per 100 cc. Present	mg. magnesium per 100 cc. Found
1·25	1·22
	1·23
2·50	2·46
	2·47
5·00	4·95
	4·93

(2) Found in serum

	mg. magnesium per 100 cc.
I.	2·41
II.	2·38
III.	2·49
IV.	2·40
V.	2·52

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LXXIII. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

I. CARBOHYDRATE METABOLISM. PRELIMINARY PAPER.

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The work about to be described constitutes the preliminary portion of an investigation upon the metabolism of brain cells under conditions both normal and abnormal. The work of Warburg, Posener and Negelein [1924], who showed that brain tissue is capable of converting large amounts of glucose into lactic acid, suggested that a study of the sugar metabolism of the brain might be of considerable interest. It was therefore decided to attempt a comparison of sugar metabolism of the brain in a normal animal, and in an animal suffering from the effects of a convulsant dose of insulin. Rabbits were used for all the experiments.

When this work was already in progress, the papers of Takahashi and Asher [1924] and of Cori [1925] appeared. Takahashi dealt particularly with the total carbohydrate content of the brain, including glycogen, and Cori with the free sugar content. Both were agreed that the lowering of the blood sugar by insulin had very little effect upon the content of reducing substance in the brain.

Takahashi estimated glycogen and "other carbohydrate" (the latter being extracted by a modification of Meyerhof's method) in rabbit brains. The values for reducing substance were obtained by the Folin-Wu technique. His average value for normal animals was: glycogen 38·6, "other carbohydrate" 39·6 mg. %. For insulin-treated animals, prior to convulsions, the average values were: glycogen 46·5, "other carbohydrate" 42·5 mg. %; for "convulsed" animals, he gives three sets of values: glycogen 27, "other carbohydrate" 8; glycogen 6, "other carbohydrate" 41; glycogen 6, "other carbohydrate" 38 (all mg. %).

Cori treated his extracts of rabbit brain with Lloyd's alkaloidal reagent, with the object of removing reducing substances other than glucose, and then estimated the remaining reducing substance by the method of Hagedorn and Jensen. His figures for normal animals (used as controls) range from 43–25 mg. %. He gives only a few values for rabbits, and the variations between them are large, yet length of time of starvation seems to be the only factor determining the selection of "control" animals, since these do not correspond with the "experimental" in age or weight.

We were able to confirm the fact that no alteration in the amount of reducing substance in the brain need necessarily have taken place at the onset of insulin convulsions. The actual figures we obtained were, however, higher than those given by the two authors mentioned. It is therefore thought advisable to give an account of the methods employed in this work.

The rabbit is killed by a blow on the neck, its brain removed as quickly as possible, and, where an immediate determination is to be carried out, the brain is then placed in a beaker surrounded by freezing mixture, chopped whilst very cold, and weighed out when frozen hard. Whilst still frozen it is placed in 20 % trichloroacetic acid (or alternatively in absolute alcohol). The removal of the brain can usually be accomplished in four or five minutes.

Two methods of extraction were employed, and where possible were both carried out on the same brain.

I. Extraction with trichloroacetic acid (20 or 10 %), a method suggested to us by Mr H. D. Kay. The tissue is ground with sand and ice-cold trichloroacetic acid, allowed to stand for some minutes at 0°, and filtered through muslin. This process is carried out three times. The cloudy solution thus obtained is centrifuged (filtration being very slow or practically impossible), the supernatant fluid poured off, and the precipitate washed three times before being discarded. The beakers, muslin, etc., are also well washed. The solution is next neutralised. It is usually still faintly cloudy, but a final centrifuging after neutralisation leaves it absolutely clear. The reducing substance present in this clear solution is estimated by the method of Hagedorn and Jensen [1923].

II. Extraction with alcohol. The tissue is allowed to soak in 90 % alcohol for some minutes to stop all enzyme action. It is then filtered off through muslin, ground up, placed in 60 % alcohol and left overnight. Next day the tissue is filtered off, ground, soaked for an hour in 60 % alcohol and finally filtered. The process is repeated once more. The cloudy filtrate is centrifuged till it is as clear as possible, the supernatant fluid poured off, and the precipitate washed three times before it is discarded. The solution is next heated gently on a water-bath until all the alcohol has disappeared; it is then full of a flocculent precipitate. Colloidal "iron" is added until the solution is clear; the addition of a little sodium sulphate seems to ensure more successful precipitation by the iron. The solution is filtered while still warm and the bulky iron precipitate is removed from the filter-paper, washed and filtered through the same paper. The washing is carried out three times. The reducing substance in the clear fluid thus obtained is estimated by the method of Hagedorn and Jensen. The estimation is usually done in triplicate, sometimes in quadruplicate.

The clear solutions obtained by these two methods obviously contain no substances that are not soluble in water. It is unlikely that any breakdown of larger molecules either by enzyme action or by acid hydrolysis could occur during the course of the extraction processes under the conditions described.

The actual values obtained are shown in Table I. The values given by the alcohol extraction method are always higher than those given by the trichloroacetic acid method. The individual variation is obviously considerable. As the two methods show a definite parallelism it seems unlikely that this variation is due to any error of technique.

Glycogen estimations have not as yet been attempted.

Table I.

No.		Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcoholic method. Reducing sub.	
				Unhydrolysed mg./100 g. brain	Hydrolysed mg./100 g. brain
	Normal				
1	Young	3	100	122	257
2	,,	4	113·8	123·4	216
3	,,	3	109·15	121·3	228
11	,,	2·5	89·1	117	176
13	,,	3	84	117·26	180·6
15	,,	3	76·8	—	189
6	Adult	4	70·6	94·8	152·6
8	,,	4	77·6	—	—
	Insulin				
4	Young	3·5	78·7	93·4	196
5	,,	3	80·03	89·95	186
7	Adult	4	69·5	94·7	146·3
9	,, (C)	4	62·8	84·7	149
10	,,	4	—	90·7	159·4
14	Young	2·5	81·47	104·8	188·2

"C" = convulsed.

The reducing values always rose considerably on hydrolysis, thus indicating the presence of some disaccharide, or polysaccharide, or of some hydrolysable compound yielding a reducing substance.

It will be seen that on an average the rabbits which had received doses of insulin gave somewhat lower values than the normal rabbits, and one or two of the lowest values were given by the former group. It does not seem, however, that this fall in the content of reducing substance always occurs, and it is therefore not likely to be of much importance in accounting for the occurrence of the convulsions.

The extent of the individual variation made comparison of the normal and "insulin" rabbits very difficult. We found, however, that rabbits taken from the same litter gave very constant results, and we adopted the plan of taking both experimental and control animals from the same litter. The figures in Table II show that much clearer results can be obtained in this way; they do not indicate any fall in the amount of reducing substance as a result of treatment with insulin.

To avoid confusion with any possible result of the convulsive muscular movements themselves, the "insulin" rabbits were usually killed in the flaccid condition which immediately precedes the actual convulsions.

Table II.

No.	Litter A	Wt. brain taken g.	Trichloroacetic acid method. Reducing sub. mg./100 g. brain	Alcohol method. Reducing sub. Unhydrolysed mg./100 g. brain	Alcohol method. Reducing sub. Hydrolysed mg./100 g. brain
13	Young normal fed	3	84	117·26	180·6
14	Young insulin starved	2·5	81·47	104·9	188·2
15	Young normal starved	3	76·8	—	189·47
	Litter B				
	Young insulin stood (C)	3	—	145·5	207·3
	Young normal stood	3	—	127·7	187·3
	Young insulin (C)	3	—	137·3	205·6
	Young insulin	3	—	146·88	208·1

"C" = convulsed.

"Stood" indicates that the brain was allowed to stand for 24 hours at room temperature before extraction.

In view of the fact that brain tissue is capable of breaking down large quantities of glucose, and considering also that the convulsions coincide with a low blood sugar, and can be cured by administration of glucose, it seems curious that the reducing substance in the brain tissue should be left untouched when the blood sugar is low.

One possible explanation which suggested itself was that the reducing substance which is extracted from the brain was not glucose.

In order to test this, estimations of the lactic acid content of brain tissue immediately after removal of the brain were carried out, and the figures compared with those given by estimations of the lactic acid content of brain tissue after incubation or standing at room temperature. The method used was that of Meyerhof [1920]. It was necessary to substitute centrifuging for the original filtration through filter-paper. The values given by the distillations are quoted without correction, as comparative rather than absolute values were required.

The values for lactic acid in the brain immediately after death ("resting" lactic acid), expressed as mg. per 100 g. brain tissue, are all very much alike. These values are given in Table III.

Table III.

No.	Rabbit	mg. lactic acid per 100 g. brain	Description	
9 A	Normal	65·29	Resting	Halves of same brain
9 B	,,	62·74	Stood 24 hours room temp. unchopped	
14	,,	98·18	Resting	Animals of same litter
15	,,	84·15	Stood 24 hours room temp. chopped	
18 A	,,	88·73	Resting	Halves of same brain
18 B	,,	85·79	Stood 24 hours room temp. unchopped in N_2	
19 A	,,	73·61	Resting	,, ,,
19 B	,,	86·18	Stood 24 hours room temp. chopped in cyanide Ringer p_{H} 8·4	
17 A	Insulin	35·29	Resting	,, ,,
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2	
20 A	,,	20·90	Resting	,, ,,
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer p_{H} 8·4	

As far as we could discover, the lactic acid content did not rise on incubation or on standing at room temperature. The brains were either allowed to stand unchopped in the air, or chopped in ordinary Ringer solution containing cyanide (*N*/65 and in some cases *N*/650 KCN).

In one case Ringer solution of p_H 8·4 was used in order to favour the formation of lactic acid as far as possible. It was usually assumed that a whole or half-brain standing unchopped in the air would be lacking in oxygen, in which case any lactic acid formed would not be oxidised. With the intention of testing this point two experiments were carried out in which the half-brains not used for "resting" determinations stood for 24 hours in an atmosphere of nitrogen.

In no case was any detectable rise in the lactic acid content of the brain tissue found as a result of standing or incubation, except possibly in the two half-brains (Table III, 19 B, 20 B) which stood chopped in alkaline Ringer solution. Even here, the rise was so slight as to be almost within the limits of error.

It seems more than probable that, during the life of the animal, glucose diffuses into the brain from the blood, and is present in the brain in estimable amounts, but that in the few minutes elapsing between the death of the rabbit and the freezing of the brain, practically the whole of this is metabolised. Some of this glucose would thus appear as "resting" lactic acid.

When the tissue is allowed to stand unchopped, or to stand chopped, in a Ringer solution of about the p_H of the body, the lactic acid content does not rise above the "resting" level. If it stands chopped in a Ringer solution of p_H 8·4, a trace of additional lactic acid seems to be formed. Probably, therefore, a small amount, but only a very small amount, of glucose remains in the brain after the death of the animal, in which case, a trace, at most, of the reducing substance estimated in the brain after death is glucose.

We have, as yet, had no opportunity of studying the fate of the reducing substances when the brain is kept at p_H 8·4, but the conclusion that there is no further formation of lactic acid on standing is supported by the fact that neither a decrease of the reducing substance in the brain tissue on incubation or on standing, nor any hydrolysis of the hydrolysable fraction can be shown to occur. To confirm this an experiment was performed using two halves of one brain for sugar estimations and two halves of another brain for lactic acid estimations (see Table IV). The two rabbits were of one litter, and the experiments were carried out on the same day. One-half of each brain was used as quickly as possible, in this case about 6 minutes after the death of the rabbit; the other half of each brain was, as usual, covered over to prevent drying, and then left unchopped for 24 hours at room temperature.

If, on the other hand, glucose is supplied to the brain tissue, that is to say if the tissue is chopped and placed in Ringer solution of p_H 7·4 containing glucose but no phosphate, considerable amounts of lactic acid are formed (see Table V).

Table IV.

			mg. per 100 g.
Rabbit 9	9 A	Lactic acid. "Resting" value	65·29
	9 B	Lactic acid. Value after 24 hours room temp. ...	62·74
Rabbit 10	10 A	Reducing substance. "Resting" value; unhydrolysed	107·3
	,,	Reducing substance. "Resting" value; hydrolysed ...	165·7
	10 B	Reducing substance. Value after 24 hours room temp.; unhydrolysed	105·6
	,,	Reducing substance. Value after 24 hours room temp.; hydrolysed	169·8

Reducing substance calculated as glucose.

Table V.

No.		mg. lactic acid per 100 g. brain
8 B	Stood 24 hours room temp. in Ringer (N/65 KCN)	76·60
8 A	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	207·70
21 A	Stood 24 hours room temp. in Ringer (N/65 KCN)	62·70
21 B	Stood 24 hours room temp. in Ringer (N/65 KCN) + 0·25 % glucose	293·30

In every case p_H of Ringer = 7·4.

It seemed that some knowledge of the actual reducing substances present would be very useful at this point, but so far the information obtained has been somewhat scanty, and the matter is still under investigation.

Preliminary experiments indicate the presence of a definite amount of pentose in the aqueous solution obtained after alcoholic extraction (and probably also after trichloroacetic acid extraction) of the tissue. The pentose tests are given very strongly by the solution, whereas Van der Haar's [1918] test, said to be specific for glycuronates, is negative. The method described by Tollens [1910] for the estimation of pentoses, has so far been attempted in one case only. The value obtained in this one case was about 20–30 mg. pentose in 100 g. brain tissue. That such an appreciable amount of pentose is present in our solutions is surprising, since the pentose or pentose-containing substance estimated must be soluble in water, and it is difficult to see how any hydrolysis of substances such as nucleic acids could occur during the extraction process. Work bearing on this question is still continuing.

We have so far no information regarding the nature of the hydrolysable substance. Estimations of the inorganic phosphates before and after hydrolysis have been carried out, and seem to show that the hydrolysable substance is not a phosphate compound, since there is no parallel rise of inorganic phosphates on hydrolysis. Again, the numerical relationship of the reducing values before and after hydrolysis (the method of Hagedorn and Jensen being used throughout) is not such as would be found were the hydrolysable substance a disaccharide such as maltose or lactose. By the Hagedorn and Jensen method maltose and lactose are estimated as about 75 % of their weight of glucose—much higher than they are estimated by copper reduction methods.

Actual figures are as follows:

Amounts of maltose and lactose estimated as 75 mg. before hydrolysis are estimated after hydrolysis as 102 and 91·4 mg. respectively.

Reducing substance from brain (alcohol method). Estimated before hydrolysis as 84·7 mg., and after hydrolysis as 144·9 mg.

Thus the rise in the reducing value after hydrolysis of the substance extracted from brain is far larger than the rise in value of maltose and lactose after hydrolysis.

The hydrolysable substance is also extracted by the trichloroacetic acid method, but the formation of chloroform from the trichloroacetic acid on boiling makes hydrolysis of solutions containing much of this acid inconvenient.

Finally, a comparison was made between the "resting" lactic acid values of normal and "insulin" brains, and also between the values obtained after incubation or standing. It will be seen that the "resting" values of the "insulin" brains are much smaller than those of the normal brains (see Table VI). This supports the view that there is a shortage of glucose in the brain of a rabbit which is on the verge of insulin convulsions.

It is, however, always possible that the low values are due to an increased oxidation of lactic acid as a result of the administration of insulin. It is hoped that this question will be decided by a study of the relationship between the level of glucose in the blood and the amount of lactic acid in the brain after death.

Table VI.

No.		mg. lactic acid per 100 g. brain	Remarks
5	Normal	90·16	Resting
9 A	,,	65·29	,,
14	,,	98·18	,,
18 A	,,	88·73	,,
19 A	,,	73·61	,,
19 B	,,	86·18	Stood 24 hours room temp. $N/650$ KCN Ringer p_H 8·4
1	,,	72·74	Stood 24 hours room temp. chopped; cyanide Ringer
2	,,	85·53	Stood 24 hours room temp. unchopped
4	,,	75·78	Stood 3 hours room temp. unchopped
8 B	,,	76·60	Stood 24 hours room temp. chopped in cyanide Ringer
9 B	,,	62·74	Stood 24 hours room temp. unchopped
15	,,	84·15	Incubated 4 hours unchopped
18 B	,,	85·79	Stood 24 hours in N_2 at room temp. unchopped
6	Insulin	38·70	Resting
7	Insulin (C)	28·16	,,
17 A	Insulin	35·29	,,
16	,,	42·70	,,
20 A	,,	20·90	,,
17 B	,,	21·48	Stood 24 hours room temp. unchopped in N_2
20 B	,,	24·64	Stood 24 hours room temp. chopped in cyanide Ringer

Averages

Normal		Insulin	
Resting	Stood or incubated	Resting	Stood or incubated
83·19	78·66	33·15	23·06

"C" = convulsed

It appears from these preliminary experiments that the reducing substance in brain tissue which is estimated by our methods, and probably, also, by the methods employed by Takahashi and Cori, is not glucose. The brain is therefore mainly dependent on the blood circulating through it for its supply of glucose; and when the blood sugar level has fallen low, the brain may well be supposed to suffer from glucose starvation. The fact that the reducing substance found in the brain is not necessarily abnormally low at the onset of the convulsions is not significant, since this reducing substance is not glucose.

SUMMARY.

1. The amount of reducing substance which can be extracted from the brains of normal rabbits, and from those of rabbits treated with insulin, has been investigated. Two different methods have been used simultaneously for extraction, and reducing substance has been estimated by the method of Hagedorn and Jensen.

2. It is concluded that there is no marked change in the amount of reducing substance as a result of insulin administration. This confirms the findings of Cori and of Takahashi.

3. The reducing substance of brain is not capable of giving rise to the formation of lactic acid, although, in similar conditions, abundance of lactic acid is formed by the brain from added glucose. Further, the reducing substance consists partly of a pentose, and partly of a substance, the reducing power of which is greatly increased after acid hydrolysis. Probably little or none of it is glucose.

4. Determinations of "resting" lactic acid on the brains of normal and of "insulin" rabbits show a greatly reduced lactic acid formation in the latter case. Neither in "normal" nor in "insulin" brains is there an increase in lactic acid formation over the "resting" value after standing or incubation at body p_H.

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LXXIV. THE PERSISTENCE OF VITAMIN A IN PLANT TISSUES.

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It was recognised at a very early stage of research on vitamin A that those plant tissues which have been engaged in the process of carbon assimilation contain relatively large quantities of this factor and that resting tissues such as seeds contain very small quantities, if any, of it. Its absence from etiolated leaves (*i.e.* those which have never been exposed to light) has been shown, and also its presence in all plant tissues so far examined which contain lipochromes and which have also been exposed to the light. Of underground structures, the only ones found to contain more than the merest trace are the carrot and the yellow sweet potato, each of these also containing lipochromes [Steenbock and Gross, 1919; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922; Coward, 1923, 2]. Comparisons between different green vegetables or salads have sometimes been drawn with regard to the vitamin content, but apparently very little consideration has been paid to the possible differences of vitamin content of leaves of the same plant grown or preserved under different conditions. Hart, Steenbock, Hoppert and Bethke [1922] drew attention to a difference in vitamin content of two different samples of hay but ascribe it to the method of curing rather than to a difference in the hay on cutting. Drummond and his co-workers [1924] had also noticed a very marked difference in the vitamin A content of butters made from the milk of cows fed at the beginning of the experiment on a sample of hay which had been cut when growing vigorously and, later, on one which had been cut when it was beginning to wither. That this difference in the butters could be ascribed largely to a difference in the vitamin content of the two samples of hay was shown in the results of a feeding test of the hays themselves on rats. An isolated observation by Coward and Drummond [1921] that shoots of *Tradescantia* kept in water-culture solution in the dark for some 8 weeks had lost only very little of their vitamin activity, and a further observation by Coward [1923, 1] that sunflower seedlings exposed to the light and then kept in the dark for (*a*) 1, (*b*) 8 days had apparently lost none of their activity, seemed to make it desirable to investigate the question of the persistence of vitamin A in plant tissues more fully. It was decided to use wheat seedlings for this purpose because they could be grown easily under laboratory conditions and also to compare green and yellowing autumn leaves, for supplies of these growing under natural conditions could easily be obtained.

EXPERIMENTAL.

Wheat seeds (Carter's Red Stand-up) were set in pots on successive days so that seedlings of the same age should be available for feeding on each day of the test. They were kept in the dark until 8 days old, transferred to the light as the experiment demanded and the shoots cut between 9 and 9.30 a.m. each morning. These were carried in a covered box to the animal room and were eaten immediately by the rats. The basal diet was that in general use in these laboratories. It consisted of:

Caseinogen, heated in thin layers 24 hours, 105°	20 g.
Starch (rice)	50 ,,
Hardened bean seed oil	10 ,,
Salt mixture	5 ,,
Yeast extract	5 ,,
Lemon juice	5 cc.

The daylight to which the shoots were subjected was never very brilliant, the experiment being carried out in July and August of 1924. It did not vary much from day to day, but any variations would be felt equally in the different experiments as they were all run concurrently with this purpose in view. At first the test was carried out on one shoot, then the dose was doubled and finally three shoots were used to obtain continued growth.

A. *Etiolated shoots.*

Eight days after the seeds were set, the shoots had grown to a height of about 3 inches. No evidence of even traces of vitamin A was obtained by giving one, two or three shoots to each test rat per day (Fig. 1 *a*).

B. *Effect of one day's exposure to light.*

Each day a pot containing 8 days old seedlings was exposed to such daylight as there was for 1 day. The shoots turned green and produced a small amount of vitamin A as shown in the curves of Fig. 1 *b*.

C. *Effect of absence of light after exposure to it.*

The pot of seedlings which had been exposed to light for 1 day was brought back into the dark room.

(1) First 4, then 8 and later 12 shoots were cut off close to the root and distributed equally between four small test tubes each containing 1 cc. tap water, the cut ends only standing in the water. After 24 hours the water from each test tube was given to a test rat by a pipette to see whether the vitamin had diffused into the water. The curves in Fig. 1 *c* show that there was apparently no diffusion.

(2) The shoots from the above experiment were given to other rats which only showed really satisfactory growth when the highest dose of three shoots was given (Fig. 1 *d*).

(3) Shoots which had been left on the plants in the pot during 24 hours in the dark were also tested, and caused resumption of growth in rats, though not such a satisfactory response as was given by the shoots which had been standing in water. It is doubtful whether the difference is great enough to justify a conclusion that the vitamin had travelled down to the living root more readily than it had diffused into the water (Fig. 1 *e*).

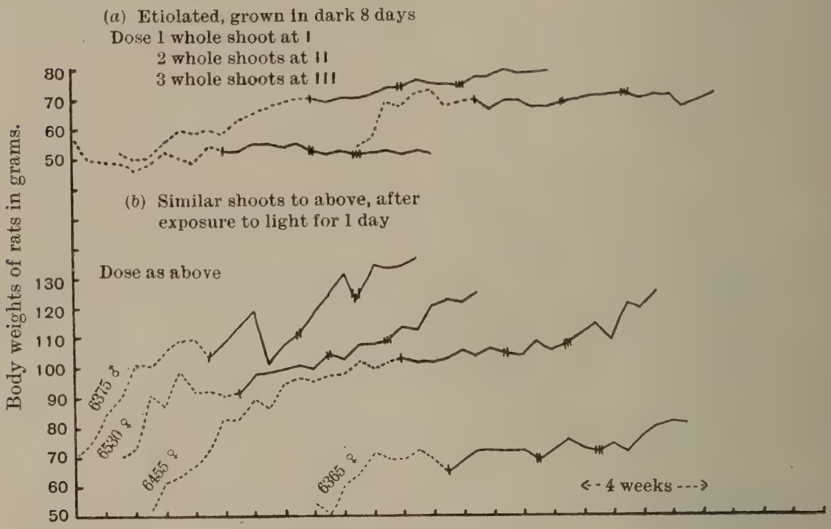

Fig. 1 *a*, *b*. Wheat seedlings.

(4) Other shoots of the same series were tested after the plants had been kept for 16 days in the dark. During this time, they had grown to about three times their original length, a second leaf had grown out of each shoot and the whole had become nearly colourless. A little green colour was left in the oldest leaf, and though the tips of the leaves had become dry and withered, for the most part the leaves were still fairly fresh. That the vitamin had not been destroyed during this time is shown in Fig. 1 *f*.

D. *The vitamin content of the withered leaf.*

A parallel series of seedlings was set and grown in the open air and daylight. The first-formed leaf was tested when the plants were 13 days old and was found to give growth. Similar first-formed leaves were tested when they were 43 days old and quite dried up and withered. They gave no growth (Fig. 2 *a* and *b*).

Hence it appears that the vitamin is not used up in any process carried on in the dark, and is not destroyed until the leaf really dies.

Confirmation of this point was sought in a comparison of the green and yellowing autumn leaves of various trees, *e.g.* lime, plane and poplar, and also of watercress and cabbage. The tree leaves that were used, both green and yellow, were collected fresh each day from those that had been blown down during the previous day or two. The greenest parts were used for the "green" test, the yellowest (fresh) parts for the "yellow" test. The dead, withered leaves of the lime were also tested. The watercress supply was renewed twice

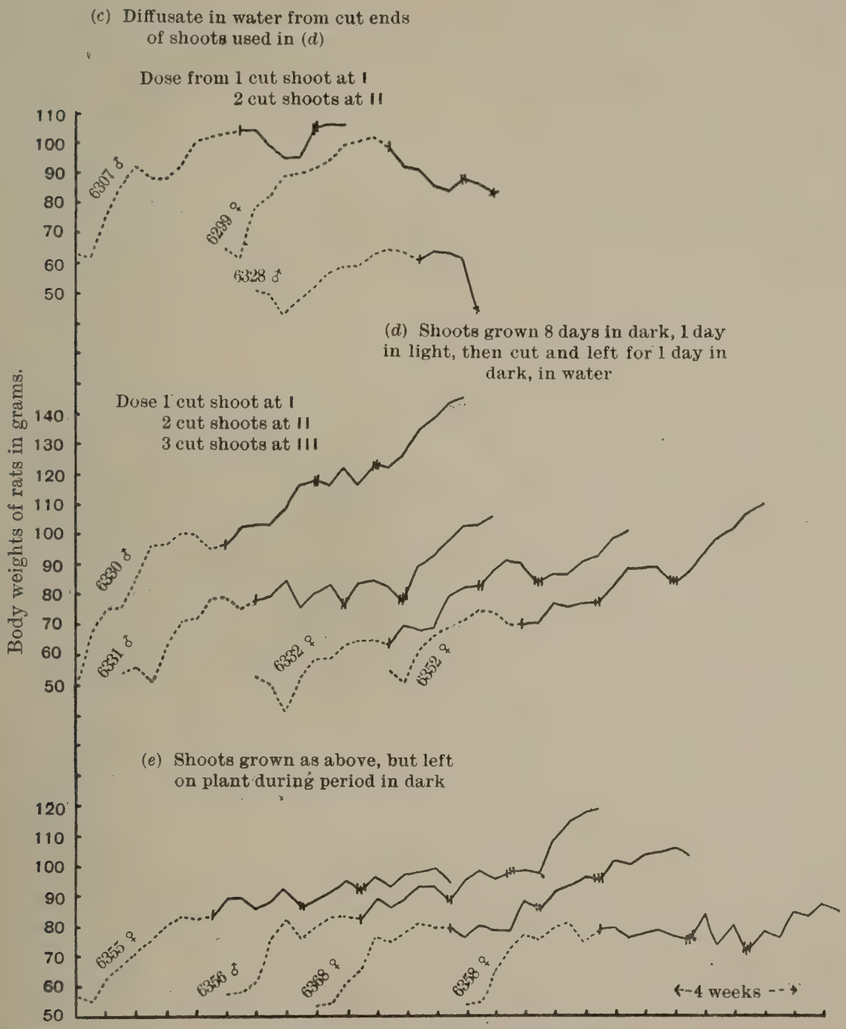

Fig. 1 *c*, *d*, *e*. Wheat seedlings.

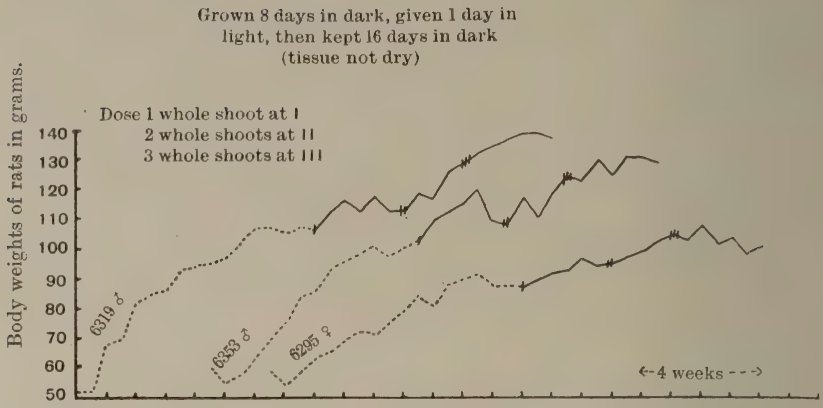

Fig. 1 *f*. Wheat seedlings.

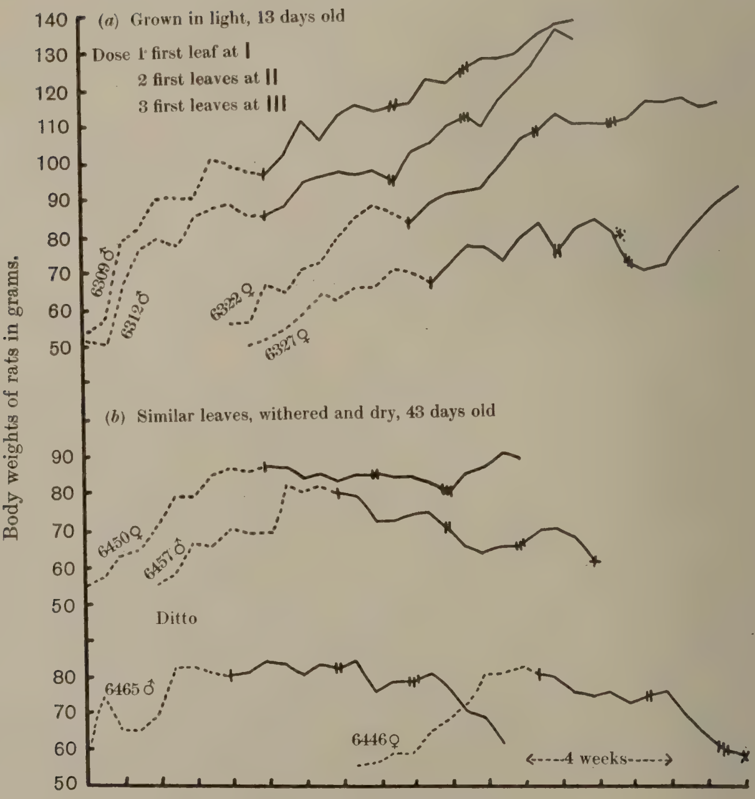

Fig. 2. Wheat seedlings.

* This rat had both legs broken accidentally about this date, which may have accounted for a temporary fall in weight.

a week and a comparison made between the fresh green leaves of the shoots and the yellow ones that had changed colour since coming into the laboratory, during which time the shoots had been freely supplied with fresh tap water. In the case of the cabbage, the comparison was made between the strong green outer leaves and similar leaves that had turned yellow on the plant while its stem was standing in water in the laboratory. It was necessary to renew this supply twice a week in order to ensure the testing of yellowed leaves that had grown under the same conditions as the green ones. In view of the fact that light is a controlling factor in the formation of vitamin A, the tests were carried out with as nearly as possible equal *areas* of leaf surface, *i.e.* about 3 square inches daily, rather than equal *weights*.

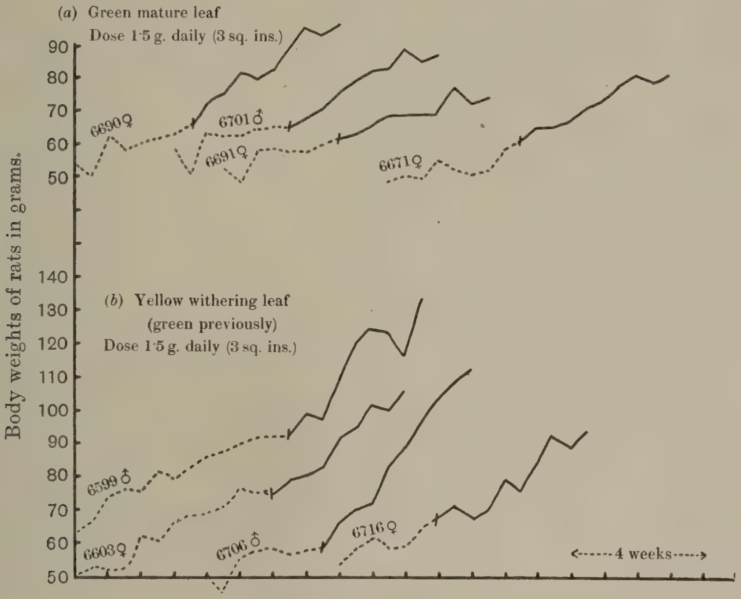

Fig. 3. Cabbage leaf.

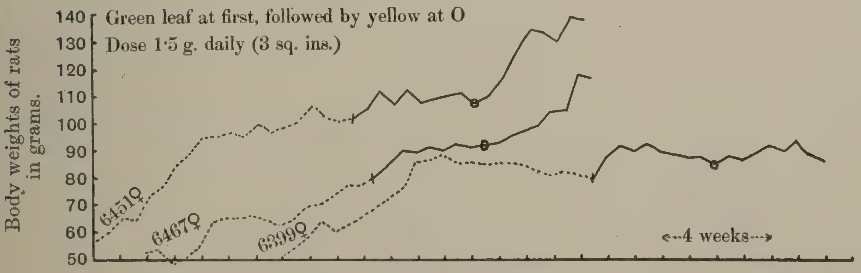

Fig. 4. Plane leaf.

Two typical results of these feeding tests are seen in Figs. 3 and 4. In each case the growth obtained with the yellow leaves was at least as good as that obtained with the green; it appears to be even better, but with such small numbers of animals in each test it is difficult to say whether the observed difference has any real significance. The result with the plane leaves, however, appears to be quite definite.

Withered lime leaves which had passed through the clear yellow stage, fallen from the tree and died, gave no growth.

Thus, it would appear that vitamin A is not used up in the yellowing autumn leaf until it actually dies.

One test was carried out on the calyx cups of the winter cherry (*Physalis Alkekengi*). The specimens used had been picked when quite fresh, dried and kept for 12 months. A dose of 0·1 g. per day was sufficient to cause resumption of growth in test animals. A similar test made previously [Coward, 1923, 2] on a different sample immediately after drying had given good growth also on a dosage of 0·1 g. daily.

It is interesting to examine these results in the light of the work of Steenbock and Nelson [1923] on the necessity of the antirachitic factor for growth. No attempt was made to supply the test rats with the antirachitic factor other than whatever may have been present in the shoots, but it may safely be assumed that where continuous growth was obtained (as with the three shoots in many cases), particularly after a long preparatory period (whether this was intentional or not), it must have been the growth-promoting vitamin A which the shoots supplied.

SUMMARY.

1. Vitamin A is not used up in any process carried on by living plant tissue in the dark.

2. It appears to increase when a leaf loses its green colour and becomes yellow.

3. It is completely destroyed when the leaf dries up and dies.

4. It does not diffuse appreciably into water from the cut ends of shoots.

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LXXV. STUDIES ON XANTHINE OXIDASE.

V. THE FUNCTION OF CATALASE.

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(*Received May 1st, 1925.*)

Although the enzyme catalase has been known for a considerable time, its biological function has never been definitely settled. It was first suggested by Loew [1902] that it had a protective action, and that its function was to prevent the accumulation in the cell of the hydrogen peroxide formed during the oxidation processes connected with cell respiration. It is well known that hydrogen peroxide is extremely toxic to most enzymes, and its presence in the cell, except in very small concentrations, would rapidly bring about their destruction. Loew suggested that the presence of catalase prevented this by keeping the peroxide concentration down to a low level. This view has been fairly generally held, but so far without having been established by definite experimental evidence except in the case of certain bacterial systems. McLeod and Gordon [1922] showed that pneumococci with a free supply of oxygen produced hydrogen peroxide in amounts sufficient to inhibit their growth; but if catalase was added they were able to grow much more vigorously than in its absence, so that Loew's view is thus supported at any rate as far as bacteria are concerned. In the present paper evidence is given to show that catalase does actually have a protective function in animal tissues.

In the second paper of this series [Dixon and Thurlow, 1924, 2] the results have been given of investigations on the xanthine oxidase by means of the methylene blue technique. Morgan, Stewart and Hopkins [1922] also carried out a number of experiments on this enzyme by measurements of oxygen uptake, and it was during an attempt to extend these investigations that the facts described in the present paper came to light.

The preparation of xanthine oxidase previously described [Dixon and Thurlow, 1924, 1] was employed for the investigation. It is very suitable for the purpose, as it probably contains no other enzymes and shows no oxygen uptake in the absence of hypoxanthine, xanthine, etc. The oxygen absorption was measured by means of the Barcroft differential apparatus of the type used by Morgan, Stewart and Hopkins. The flasks of this apparatus were of a flattened form which exposed the fluid in a thin layer to the air above it so as to allow very rapid diffusion of oxygen into the liquid. Under these conditions the rate of oxygen absorption depends only on the rate at which it is used up by the enzyme, and is not limited by the rate of diffusion into the liquid. In setting up each experiment solutions of the same composition were added to both flasks, but the hypoxanthine was added to one flask only. This ensured that each experiment was properly controlled. The experiments were in all cases carried out at a temperature of 40° and at p_H 7·6.

On investigating the effect of a change in the concentration of the enzyme on the rate of oxygen uptake, using a constant amount of hypoxanthine, it was found that the rate of the reaction was *not* directly proportional to the enzyme concentration, as was found to be the case with the methylene blue technique. Moreover, the total amount of oxygen taken up by the system was not always independent of the enzyme concentration. As long as the xanthine oxidase is present in excess the total oxygen uptake corresponds with the calculated amount necessary to oxidise the hypoxanthine (or xanthine) to uric acid. When, however, the concentration of enzyme used in the experiment is reduced, a point is reached where the oxygen uptake varies with the amount of enzyme taken. These facts can be seen from Fig. 1, where the results of a typical experiment are given.

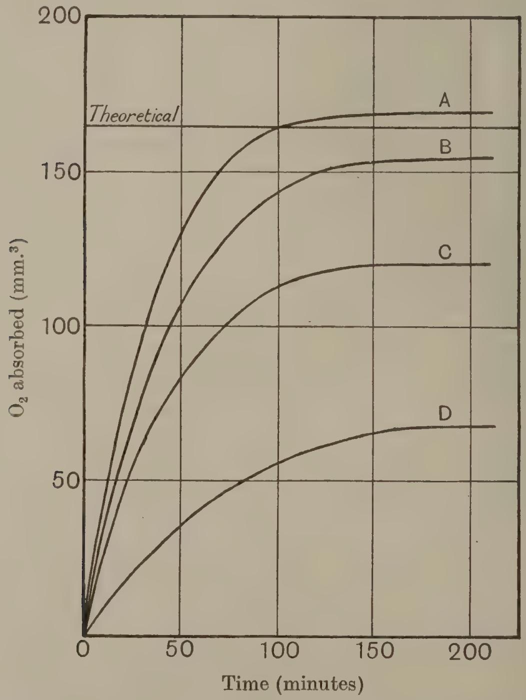

Fig. 1. Oxygen absorption by hypoxanthine with varying amounts of xanthine oxidase. The solutions were made up as follows:

	A cc.	B cc.	C cc.	D cc.
Oxidase solution	2·0	1·5	1·0	0·5
p_H 7·6 buffer	0·5	1·0	1·5	2·0
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

The curves suggest that the enzyme is being gradually destroyed during the course of the reaction. In order to test whether this was the case the contents of the flasks of the Barcroft apparatus were transferred at the conclusion of the experiment to vacuum tubes, and tested anaerobically with methylene blue and a little fresh hypoxanthine. By comparing the reduction-time given by an enzyme solution which has brought about the oxidation of the hypoxanthine with that given by the solution in the control flask of the same apparatus it is possible to determine approximately how much of the enzyme has been destroyed during the oxidation. By this method it was found that in experiments B, C and D the enzyme had been totally destroyed during the oxidation, whilst in experiment A there had been considerable, though not complete, destruction. It follows therefore that in order to make sure that the oxidation of the hypoxanthine shall proceed to completion it is necessary to use an amount of enzyme sufficient to ensure that some of it shall be left undestroyed at the end of the reaction.

The experiment given in Fig. 1 was repeated, using milk instead of the xanthine oxidase preparation, and similar results were obtained. It is worth noting that the amounts of milk used by Morgan, Stewart and Hopkins in their experiments were not very greatly above the minimum required for complete oxidation of the hypoxanthine, so that it is almost certain that the greater part of the oxidase had been destroyed by the end of their experiments.

These facts show that it is almost impossible to interpret curves of oxygen absorption in terms of enzyme dynamics, since the concentration of enzyme is continuously changing during the progress of the reaction. The oxygen-absorption method is therefore not well suited to a quantitative investigation of the dynamics of systems of the type of the xanthine oxidase except for special purposes, and it seems that other methods, such as the methylene blue technique, are more likely to yield trustworthy results.

It can be shown from the methylene blue experiments given in the earlier paper [Dixon and Thurlow, 1924, 2] that the enzyme is not destroyed during the course of the reaction when methylene blue is substituted for oxygen. The effect seems only to occur in the presence of oxygen, and this fact at once suggests the possibility that hydrogen peroxide may be responsible for the destruction. It has been shown by Thurlow [1925] in the preceding paper of this series that hydrogen peroxide is formed during this reaction in appreciable quantities. The writer has found that xanthine oxidase is very sensitive to hydrogen peroxide, and is quickly destroyed even by quite small concentrations. It follows that the destruction of the enzyme during the reaction is indeed to be expected.

We have then in the xanthine oxidase a system, normally occurring in animal tissues, on which catalase should, if Loew's view is correct, exert a protective action. In order to investigate this question experimentally it is desirable to obtain a preparation of catalase which is free from xanthine oxidase and from oxidisable substances. Several methods of preparation were tried, and the one which was found to give the most satisfactory results in this respect was that of Batelli and Stern [1904]. Catalase preparations made from liver by this method were found not to take up oxygen or reduce methylene blue either in the presence or in the absence of hypoxanthine.

The liver was minced and extracted with twice its own volume of water. The extract was mixed with two volumes of alcohol, and the resulting precipitate filtered off and dried. This precipitate was then extracted thoroughly with three volumes of water with shaking for some hours, and the extract after filtering was mixed with three times its volume of alcohol. The not very voluminous precipitate thus produced was filtered off and dried *in vacuo*. The preparation is readily soluble in water, and in the dry form retains its activity very satisfactorily.

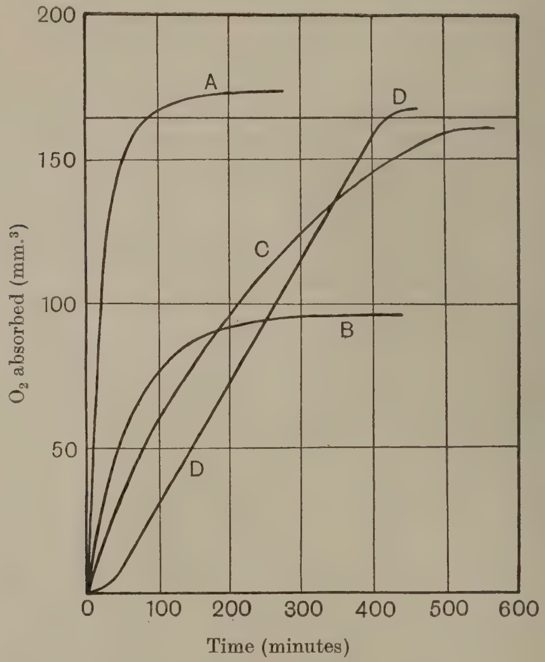

Fig. 2. Effect of addition of catalase.

The solutions were made up as follows:

	A	B	C	D
	cc.	cc.	cc.	cc.
Oxidase solution	2·0	0·5	0·5	0·5
Catalase solution	—	—	0·5 (weak solution)	0·5 (strong solution)
p_H 7·6 buffer	0·5	2·0	1·5	1·5
Hypoxanthine (2 mg. per cc.)	0·5	0·5	0·5	0·5

In Fig. 2 is shown the effect on the oxygen uptake of the addition of catalase. Curves A and B show, as in Fig. 1, the course of the oxygen absorption with a fairly large and a small oxidase concentration respectively; curve C shows the absorption with the same small amount of xanthine oxidase as in curve B, but with the addition of a relatively small quantity of catalase. It will be seen that the catalase has prevented, at any rate in large measure, the destruction of the oxidase, since the reaction ultimately proceeds to completion even with the small oxidase concentration, though of course at a slower rate than with the higher concentration in curve A.

At the conclusion of the experiment the solutions were tested as before with methylene blue *in vacuo* after the appropriate additions of hypoxanthine, in order to determine how much destruction of the oxidase had occurred. The results showed that in A about 90 % of the oxidase had been destroyed, whilst in B destruction had been complete. In C, however, only about 25 % had been destroyed, and the catalase thus shows a protective action which is very marked considering the small amount added. The same experiment has been repeated with several different preparations of xanthine oxidase and liver catalase, and similar results have invariably been obtained.

If stronger solutions of catalase are used, much more complete protection of the enzyme can be obtained. Curve D shows a typical curve obtained with an amount of catalase several times as great as that used in curve C, with the same amount of oxidase as in curves B and C. It is interesting to note that when the protection becomes more complete the curve of oxygen uptake becomes practically a straight line. When very strong catalase solutions are used, the curves always show a small "latent period" effect. The reaction begins very slowly, and does not attain its full velocity for 20–30 minutes. This effect, which can be seen to some extent in curve D, is at present unexplained.

It will be seen that initially the rate of oxygen uptake is definitely smaller when catalase is present than in its absence. This effect is quite independent of the "latent period," and can be seen in curve C as well as in curve D. Of course, complications are introduced by the fact that the oxidase concentration, when no catalase is present, soon falls, so that the velocity of the absorption falls below that given by the solutions containing catalase; but we may safely assume that during the initial parts of curves B, C and D the concentration of oxidase is roughly the same in all three cases. It is therefore legitimate to compare the velocities during the first part of the experiment. Thus it appears that catalase actually lowers the rate of oxygen uptake. This inhibition is, however, only apparent, and is to be expected. The catalase decomposes hydrogen peroxide in the following way $2H_2O_2 \rightarrow 2H_2O + O_2$, and there is thus an evolution of oxygen gas. The apparent volume of oxygen absorbed, as given by the reading of the Barcroft apparatus, is then the actual volume utilised by the xanthine oxidase *minus* the volume evolved by the catalase. Any catalase action is therefore bound to diminish the observed rate of oxygen uptake. This is probably the true explanation of the phenomenon, and there seems to be no reason to suppose that catalase has any direct action on the xanthine oxidase. The above action, of course, only affects the observed rate of oxygen uptake; the amount of oxygen ultimately absorbed remains unaltered.

It was stated above that destruction of the oxidase occurred in milk as well as in solutions of the catalase-free caseinogen preparation. The amount of catalase contained in milk thus seems to be insufficient to protect the oxidase. The samples of milk used in the experiment, however, appeared to contain only a small amount of catalase, to judge from rough tests, and it is possible that a more average milk would not show so marked a destruction.

SUMMARY.

1. When the purine bases are oxidised by molecular oxygen in presence of xanthine oxidase as a catalyst, the oxidase is progressively destroyed during the course of the reaction. This fact makes the measurement of oxygen uptake unsuitable as a general method for the investigation of the dynamics of the enzyme.

2. The destruction is due to the hydrogen peroxide which is known to be formed during the reaction.

3. The destruction of the oxidase is prevented by the addition of catalase, which is thus shown to have a protective function in animal tissues.

The writer wishes to express his thanks to Sir F. G. Hopkins for his interest in the work, and also to the Royal Commissioners for the Exhibition of 1851 for the senior studentship held during the period of the investigation.

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LXXVI. BACTERICIDAL ACTION OF SOME ORGANIC COMPOUNDS OF MERCURY.

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Though many organic compounds of mercury have been prepared, especially during the last few years, and much laboratory and clinical work has been done in ascertaining the possibilities of their application as medicaments, there is as yet a scarcity of systematic information regarding the bactericidal action of such substances, although such knowledge must form the basis on which the use of these products in medicine depends.

The most important work of this kind done so far is that of Schoeller and Schrauth. They showed [1910] that, beginning with a simple compound such as hydroxymercuribenzoic acid $HO—Hg—C_6H_4.COOH$, there was little change in bactericidal activity when HO— was replaced by bromine or chlorine, a marked diminution on replacement by iodine or cyanogen, still greater reduction on replacement by sulphur, and that activity almost vanished when HO— was replaced by a second benzoyl radical as in mercuridibenzoic acid.

In a second paper [1912] they showed that by the insertion of other substituents in hydroxymercuribenzoic acid the bactericidal action could be markedly influenced. Thus it was increased by halogen or alkyl groups or by a second hydroxymercuri residue and diminished by hydroxyl, sulphonic acid, or amino groups. These observations relate for the most part to new substituents in the *ortho*-position to the carboxyl group, but some evidence was obtained that the orientation of substituents also affected the intensity of bactericidal action, *e.g.* in $C_6H_3(COONa)(Hg.OH)(CH_3O)$ the three substituents in positions 1:2:4 were less efficient than in positions 1:2:6. Two species were used in these experiments, viz. *B. anthracis* and *Staphylococcus pyogenes aureus*. There was little evidence of any specificity in action, but it must be remembered in this connection that all the compounds were not tried on each species.

In a third paper [1916] the same authors dealt with the influence of the same substituents in the absence of the carboxyl group, the latter being replaced by hydroxyl. Here bactericidal action was intensified to start with, the carboxyl group being a weakening factor, but the influence of the substituents already referred to remained the same so far as it was tried, with the addition of the nitro-group to the augmenting series. The three mercurated isomeric cresols arranged themselves in these trials in the following order of diminishing activity: *meta, ortho, para.*

Reference may also be made to Miss de Witt's work [1921] which relates mainly to the possibility of using organic compounds of mercury in tuberculosis, and covers a large number of substances ranging from mercuric chloride to complex products including chloromercuri derivatives of dyes such as methylene blue, trypan blue, methylene green, etc. The results are expressed in terms of mercury required to inhibit the growth of human tubercle bacilli on melted nutrient agar; and from the present point of view they are chiefly of interest in showing the variation in the amount of mercury (g. per litre) required for this purpose, depending on the organic or inorganic residue with which it is associated, the least effective being mercuric chloride (0·093) and the most active, acetoxymercuriallyl alcohol, $HO.CH_2CH:CH.HgO.Ac$ (0·012), chloromercurimethylene blue (0·014) and chloromercurifluorescein (0·016).

There are numerous other papers on the subject but the foregoing account is sufficient to show the general position.

It occurred to us that valuable information might be obtained by starting with a comparatively simple substance, mercurating it and a suitable selection of its derivatives and determining the bactericidal action of the resulting mercury compounds, keeping throughout to strictly comparable conditions, and using for comparison certain standard mercury compounds, which are already in more or less general use in medicine.

Of the numerous possible starting points for such an investigation we selected benzaldehyde mainly because, when we began this work, no mercurated derivatives of aromatic aldehydes had been made. The substances so far prepared have been described and their constitution discussed elsewhere [Henry and Sharp, 1922, 1924], and in the present paper only their bactericidal action need be discussed. For the elucidation of certain points it proved desirable to make a number of other organic mercury compounds, for example, those of the alkyl phenols, from which we hoped to obtain information as to the effect of replacing the aldehyde group by alkyl groups. Further, for certain clinical trials which became necessary in the course of this work, derivatives soluble in oils had to be made, and this was done by converting the hydroxymercuri compounds into salts of higher fatty acids, such as oleic, myristic, chaulmoogric, etc. These salts, which are described briefly later on, are readily soluble in almond, olive and other oils and such solutions are suitable for chemotherapeutic experiments on animals where aqueous solvents are inadvisable or useless. The bactericidal tests were all made by the methods described below, using *Bacillus typhosus*, Rawlings.

B. typhosus was selected as the test organism in preference to *B. coli* for the reasons put forward by Anderson and McClintock [1912] viz., (1) the greater reliability of the strains in the former organism, (2) the proof that the growth in the medium is actually due to *B. typhosus* and not to accidental contamination is easily made by using specific agglutination with a high titre serum.

Aqueous solutions. Most of these mercury compounds are insoluble in water but form sparingly soluble sodium or potassium salts and these were used for the tests carried out in water. The solutions were diluted to a suitable series of known concentrations with sterile distilled water and quantities of 5 cc. mixed with 0·2 cc. of a 24 hours broth culture of *B. typhosus*, Rawlings, filtered through paper. This was allowed to interact for 5 minutes at 20° and a 3 mm. platinum loopful taken into 10 cc. of sterile broth and incubated at 37° for 48 hours. The tubes showing growth in the highest concentration were tested with typhoid serum and a control serum for specific agglutination.

Serum solutions. It is now well known that the bactericidal action of many substances is reduced when they are used in presence of serum and to ascertain how this medium affected mercury compounds some trials were made with normal horse serum, which had been passed through a Berkefeld candle and heated at 58° for 2 hours. The serum in this case was used as a diluent in place of sterile distilled water as in the previous test.

Oil solutions. For the reason already stated (p. 514) it was desirable to see how the use of oil as a solvent for oil-soluble mercury compounds affected their use as bactericidal agents and these tests were carried out as follows. The reagents were diluted in sterile olive oil. 5 cc. quantities were tubed and to these varying dilutions, 0·02 cc. of the filtered broth culture was added; the tubes were then closed with sterile rubber corks and the contents vigorously shaken. After 5 minutes' interaction in a water-bath at 20° cultures were taken as before.

RESULTS OF EXPERIMENTS.

The substances are indicated by typical formulae as far as possible, *e.g.* in Table I, substance No. 1 is 3 : 5-dihydroxydimercuri-2-hydroxybenzaldehyde, which is represented thus $C_6H_2[(CHO)(Hg.OH)_2(OH) = 1 : 3 : 5 : 2]$.

From the results given (Table I) it is clear that the introduction of a mercury residue into the molecule of an organic compound enhances the bactericidal action of the latter: thus, whilst a 0·5 % solution of *m*-hydroxybenzaldehyde only kills *B. typhosus* after 4 hours' action, solutions of all the mercury derivatives of *m*-hydroxybenzaldehyde are much more active bactericidal agents, the most potent towards *B. typhosus* being hydroxymercuri-4-nitro-3-hydroxybenzaldehyde (No. 7, Table I) which kills in 5 minutes at a concentration of 0·004 %, whilst the least active are the dihydroxydimercurinitro-3-hydroxybenzaldehydes (Nos. 4 and 8, Table I) which are toxic in 5 minutes at a concentration of 0·06 %. It is noteworthy in this connection that so far from enhancing the bactericidal action, as stated by Schoeller and Schrauth, the introduction of a second mercury residue appears to reduce it, all the dimercuri derivatives of nitro-*m*-hydroxybenzaldehyde (Nos. 4, 6, 8, in Table I) being less active than the monomercuri derivatives (Nos. 3, 5, 7, 9, in Table I).

Table I. *Salts of mercurated hydroxybenzaldehydes, alkylphenols and certain related substances in water.*

No.	Substance used	Toxicity to *B. typhosus*		Mercury in toxic concentration g. %
		% kills	% fails	
Mercurated hydroxybenzaldehydes.				
1	$C_6H_2[(CHO)(HgOH)_2(OH)=1:3:5:2]$	0·0202	0·013	0·016
2	$C_6H_2[(CHO)(HgOH)_2(OH)=1:3:5:4]$	—	0·03	*
3	$C_6H_3[(CHO)(HgOH)(OH)=1:2:3]$	0·0085	0·0056	0·005
4	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:2:4:3:6]$	0·06	0·05	0·04
5	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:2$ or $4:3:6]$	0·01	0·009	0·005
6	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:2:6:3:4]$	0·02	0·01	0·013
7	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:2$ or $6:3:4]$	0·0038	0·0029	0·002
8	$C_6H[(CHO)(HgOH)_2(OH)(NO_2)=1:4:6:3:2]$	0·06	0·05	0·04
9	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:4$ or $6:3:2]$	0·0095	0·0086	0·005
10	$C_6H_2[(CHO)(HgOH)(OH)(NO_2)=1:5:4:3]$	0·117	0·095	0·06
Mercurated alkylphenols.				
11	$C_6H_2[(CH_3)(C_3H_7)(HgOH)(OH)=1:4:?:2]$	0·038	0·017	0·021
12	$C_6H[(CH_3)(C_3H_7)(HgOH)_2(OH)=1:4:2:6:3]$	0·033	0·016	0·023
13	$C_6H_3[(C_4H_9)(HgOH)(OH)=1:3:4]$	0·043	0·032	0·024
14	$C_6H_2[(C_4H_9)(HgOH)_2(OH)=1:3:5:4]$	—	0·012	*
15	$C_6H_3[(C_5H_{11})(HgOH)(OH)=1:3:4]$	0·035	0·028	0·018
16	$C_6H_2[(C_5H_{11})(HgOH)_2(OH)=1:3:5:4]$	—	0·022	*
Miscellaneous mercury compounds.				
17	Hydroxymercuri-*o*-nitrophenol†	0·0021	0·001	0·0011
18	Hydroxymercuri-*m*-nitrophenol	0·005	0·004	0·0028
19	Dihydroxydimercuri-*m*-nitrophenol	0·01	0·009	0·007
20	Hydroxymercuriacetylsalicylic acid†	0·6	0·4	0·26
21	Hydroxymercuridibromofluorescein†	0·3	0·2	0·069

* Strongest concentration possible.

† Commercial preparations issued and used as sodium salts.

In order to confirm this result mono- and di-mercuri derivatives of *m*-nitrophenol were prepared, when it was found that the dimercuri derivative (No. 19, Table I) is only half as active as the monomercuri compound (No. 18, Table I).

On arranging these results as in Table II, to show the percentage of metallic mercury in the concentration toxic to *B. typhosus*, it becomes clear that the bactericidal equivalent, thus expressed in terms of metallic mercury, depends in a marked degree on the nature of the organic residue to which it is attached.

When these effects are considered in detail it is seen that the two groups —CHO and —OH taken together are most effective in the *meta*-position to each other and least effective in the *para*-position. The entrance of a nitro-group when CHO : OH = 1 : 3, enhances the effect in position 4 (No. 7) but exerts no influence in positions 2 or 6 (Nos. 5 and 9) though it should be remembered that these compounds are not strictly comparable since the position of the mercuri-group is not the same in all three. These results differ from those of Miss de Witt [1921] with mercurated nitrophenols in which the combination $HgOH : NO_2 : OH$ was much more active than $HgOH : OH$ and was at its best with $HgOH : NO_2 : OH = 4 : 2 : 1$.

Table II.

No. in Table I	Substance	Mercury in toxic concentration g. %
17	Hydroxymercuri-*o*-nitrophenol*	0·0011
7	Hydroxymercuri-4-nitro-3-hydroxybenzaldehyde	0·002
18	Hydroxymercuri-*m*-nitrophenol	0·0028
3	2-Hydroxymercuri-3-hydroxybenzaldehyde	0·005
9	Hydroxymercuri-2-nitro-3-hydroxybenzaldehyde	0·005
5	Hydroxymercuri-6-nitro-3-hydroxybenzaldehyde	0·005
19	Dihydroxydimercuri-*m*-nitrophenol	0·007
6	2 : 6-Dihydroxydimercuri-4-nitro-3-hydroxybenzaldehyde	0·013
1	3 : 5-Dihydroxydimercuri-2-hydroxybenzaldehyde	0·016
15	2-Hydroxymercuri-*p*-iso-amylphenol	0·018
11	Hydroxymercuricarvacrol	0·021
12	2 : 6-Dihydroxydimercurithymol*	0·023
13	2-Hydroxymercuri-*p*-*tert*-butylphenol	0·024
8	4 : 6-Dihydroxydimercuri-2-nitro-3-hydroxybenzaldehyde	0·04
4	2 : 4-Dihydroxydimercuri-6-nitro-3-hydroxybenzaldehyde	0·04
10	5-Hydroxymercuri-3-nitro-4-hydroxybenzaldehyde	0·06
21	Hydroxymercuridibromofluorescein*	0·069
20	Hydroxymercuriacetylsalicylic acid*	0·26

* Commercial preparations.

Table III. *Comparative tests in serum, broth and water.*

	Medium						
	Water		Broth		Serum		
Substance	Kills %	Fails %	Kills %	Fails %	Kills %	Fails %	Mercury in toxic concentration g. %
Mercury succinimide	0·001	0·0001	0·001	0·0001	0·1	0·01	Water 0·0005 Broth 0·0005 Serum 0·05
2-Hydroxymercuri-3-hydroxybenzaldehyde 20° (time 5 minutes)	0·0085	0·005	0·05	0·01	0·05	0·01	Water 0·005 Broth 0·03 Serum 0·03
Time 24 hours; 37°	0·0005	0·0001	0·001	0·0005	0·01	0·005	Water 0·0003 Broth 0·0006 Serum 0·006
Time 48 hours; 37°	0·0005	0·0001	0·001	0·0005	0·01	0·005	Water 0·0003 Broth 0·0006 Serum 0·006

In the case of the alkyl phenols the increase of bactericidal activity due to the introduction of mercury residues is much less marked; thus whilst carvacrol, thymol, *p*-*tert*-butylphenol and *p*-*iso*-amylphenol are all toxic to *B. typhosus*, under these conditions, at a concentration of about 0·03–0·04 %, the activity of the mercury derivatives (Nos. 11–16, Table I) is of about the same order, but owing to the sparing solubility of the salts of these derivatives in water the results are perhaps not entirely trustworthy. For the same reason no conclusions can be drawn regarding the effect of replacing the aldehyde group (as in No. 2) by alkyl groups (as in Nos. 14 and 16).

It is clear from the results in Table III that there is a considerable reduction in the bactericidal action of both organic and inorganic compounds of mercury when they are used in serum in place of water: broth however has little or no effect on the activity of mercury succinimide though it reduces the activity of the hydroxymercurihydroxybenzaldehyde from about one-sixth to one-half of that shown in water.

Experiments in oil. As it is desirable for clinical work in some cases to use mercury compounds in oily media, a number of these compounds have been converted into oil-soluble derivatives by converting the —$Hg.OH$ residue into a radical containing the residue of one of the higher fatty acids. Thus if 2-acetoxymercuri-3-hydroxybenzaldehyde is heated at 100° with excess of myristic acid for 1 hour it dissolves and on extracting the cooled product with hot light petroleum (B.P. 40–60°) to remove excess of myristic acid there is left undissolved 2-myristoxymercuri-3-hydroxybenzaldehyde

$$C_6H_3(CHO)(OH)(HgO.CO.C_{13}H_{27})$$

as a colourless indistinctly crystalline powder, melting at 130° and decomposing at 200°. Found Hg = 36·44, calculated Hg = 36·56 %. This product is readily soluble in oils, especially on warming, and in like manner similar derivatives with higher fatty acids can be obtained from the various mercury compounds mentioned in Table I.

A number of such compounds have been made from 2-acetoxymercuri-3-hydroxybenzaldehyde and their bactericidal action on *B. typhosus* has been determined in olive oil. The characters of the principal compounds of this type made are as follows:

2-Chaulmoogroxymercuri-3-hydroxybenzaldehyde	$C_6H_3CHO.OH.HgO.CO.C_{17}H_{31}$	M.P. 117°
2-Lauroxymercuri-3-hydroxybenzaldehyde	$C_6H_3CHO.OH.HgO.CO.C_{11}H_{23}$	M.P. 131–3°
2-Oleoxymercuri-3-hydroxybenzaldehyde	$C_6H_3CHO.OH.HgO.CO.C_{17}H_{33}$	M.P. 110–115°
2-Capryloxymercuri-3-hydroxybenzaldehyde	$C_6H_3CHO.OH.HgO.CO.C_7H_{15}$	M.P. 130–1°

The results of the bactericidal tests in oil are as follows:

Table IV. *Higher fatty acid derivatives of 2-hydroxymercuri-3-hydroxybenzaldehyde* (No. 3, Table I).

Acid used and formula of compound	Toxicity to *B. typhosus*		Mercury in toxic concentration g. %
	% kills	% fails	
1. Caprylic acid $C_6H_3(CHO)(OH)(HgO.CO.C_7H_{15})$	0·025	0·01	0·011
2. Lauric acid $C_6H_3(CHO)(OH)(HgO.CO.C_{11}H_{23})$	0·025	0·01	0·0096
3. Myristic acid $C_6H_3(CHO)(OH)(HgO.CO.C_{13}H_{27})$	0·025	0·01	0·0091
4. Oleic acid $C_6H_3(CHO)(OH)(HgO.CO.C_{17}H_{33})$	0·025	0·01	0·0083
5. Chaulmoogric acid $C_6H_3(CHO)(OH)(HgO.CO.C_{17}H_{31})$	0·0076	0·0038	0·0026

The results of these experiments in oil indicate that the activity of 2-hydroxymercuri-3-hydroxybenzaldehyde used in the form of salts of the higher fatty acids is reduced by about half in this medium, except in the case of the chaulmoogric acid salt where the activity is doubled, due no doubt to the influence of the chaulmoogric acid, which itself exhibits considerable bactericidal action.

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LXXVII. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. I.

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(*Received March 24th, 1925.*)

It has been shown in earlier communications [Quastel and Whetham, 1924; Quastel, Stephenson and Whetham, 1925] that succinic acid readily reduces methylene blue in presence of resting or non-proliferating bacteria (*B. coli* and *B. pyocyaneus*), and that under similar conditions fumaric acid acts as a hydrogen acceptor. It has been shown, also, that these bacteria activate not only succinic, fumaric, and malic acids, but nitrates and chlorates, these substances effecting oxidations in presence of the bacteria which they do not produce at a perceptible rate in their absence.

A systematic study has now been made of the activating powers of *B. coli communis*, and this communication is intended to be the first of a series dealing with the subject. Numerous substances have been found to exert reducing and oxidising powers in presence of this organism.

Harden and Zilva showed some years ago [1915] that washed *B. coli comm.* will not reduce methylene blue and demonstrated, in a qualitative manner, that it acquired the power of reduction on addition to it of various substances incapable by themselves of effecting the reduction. Many of the substances, which they tested, will be dealt with in detail below.

The technique adopted throughout the work has been described fully in the papers quoted above. The rate of reduction of methylene blue was measured under standard conditions of temperature and hydrogen ion concentration in the presence of the organism and the substance under investigation. The conditions were always strictly anaerobic and such that growth of the organism did not occur. Sometimes the method was varied by using nitrate or chlorate as hydrogen acceptors, the amount of reduction in a given time being determined by an iodometric estimation of the nitrite or chlorite produced.

In the preparation of the emulsion of bacteria, the following points were found important. The organism was grown for 2 days on tryptic broth and separated by centrifuging; it was then washed very thoroughly by repeated centrifuging with normal saline to remove all traces of broth which is itself a powerful hydrogen donator in presence of *B. coli*. The bacteria thus washed were suspended in normal saline solution through which first air and then nitrogen were bubbled vigorously for a short time to produce a homogeneous suspension and to remove traces of easily oxidisable material. The suspension was then diluted with saline until a small volume (say 0·5 cc.) of the suspension did not reduce 1 cc. of 1/5000 methylene blue solution at p_H 7·2 and temperature 45° within several hours. In the presence of a concentration of sodium succinate equal to $M/100$, this quantity of organism was usually found to reduce the same amount of methylene blue in about 10 minutes. If the time of reduction was much shorter than this, the emulsion of bacteria was further diluted. The emulsion was kept surrounded by ice, since at room temperature there occurs a rapid diminution in activating power and a liberation of reducing substances, probably due to an autolysis of the cells. At 0° these changes are hardly noticeable during the first fortnight, and even after that proceed very slowly. A suspension of *B. coli* which had been kept in the ice-chest for 4 months still exhibited strong activating powers, and a sub-culture on agar gave a heavy growth.

It has been previously shown [Quastel and Whetham, 1924] that the reducing activity of the organism is directly proportional to its concentration, and that the reaction between succinate and methylene blue in presence of the organism has a temperature coefficient of 2·1 between 30° and 60°.

The experimental results given below are typical examples of a large number of observations. The work was done with a number of different strains and suspensions of *B. coli*, and these are referred to as *coli* 1, *coli* 2, etc. The results obtained with *coli* 1 can be compared strictly quantitatively with one another, but only qualitatively with the results obtained with *coli* 2, etc.

The substances under investigation were always brought to p_H 7·2 by means of caustic soda or hydrochloric acid.

The fatty acids.

Table I gives the results obtained with the fatty acid series.

Table I.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, and X cc. of the fatty acid solution. The volume of solution in each tube was made up to 6·5 cc. with distilled water. The reduction was carried out *in vacuo* at 45°. A control tube showed no reduction in 6 hours.

Fatty acid	X cc.	Concentration of fatty acid	Reduction time in minutes
Formic $M/20$	1	$M/130$	2·0
,, $M/2$	1	$M/13$	1·5
Acetic $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	2·8
Propionic $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	26·0
Butyric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	3·0
Isobutyric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	Not completely reduced in 3 hours
Isovaleric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	5·0

Formic acid is the most powerful hydrogen donator of the series, and with the exception of some of the sugars is the most active substance we have yet investigated. It has no reducing action *in vitro* on methylene blue, even in strongly alkaline solution.

Table II gives results with the fatty acids obtained with a different strain of *B. coli* (*coli* 2).

Table II.

Procedure exactly as described in Table I. *Coli* 2 used instead of *coli* 1. 2 cc. of an $M/2$ sol. of fatty acid were used in each case yielding a final concentration of $M/6{\cdot}5$.

Fatty acid	Reduction time in minutes
Formic	1·2
Acetic	9
Propionic	11
Butyric	30
Valeric	23
Isovaleric	25
Caproic	Not reduced
Heptylic	,,
Caprylic	,,
Nonylic	,,

Apart from formic acid, the lower fatty acids are active donators of hydrogen only at high concentrations. Caproic acid exerts a slight activity (see Table III) but higher members than this are apparently inert. A change in the activity of the organism has apparently a far greater effect on the reduction velocities due to the fatty acids than on those due to donators such as formic or succinic acids, which are active at low concentrations.

Fig. 1 illustrates the variation in times of reduction of 1 cc. of 1/5000 methylene blue solution with varying concentrations of acetic, propionic and butyric acids at p_H 7·4 and 45°, always using the same amount of organism (*coli* 1). The propionic acid curve is of a different type from those of acetic and butyric acids. In view of the oxidation of these fatty acids in the body, it is interesting that propionic acid should offer so clear a contrast to acetic and butyric acids in presence of resting *B. coli*.

The reduction time-concentration curves of acetic, butyric and formic acids (Fig. 1) exhibit a feature common to many of the hydrogen donators we have investigated, namely, a rapid decrease of reduction time up to a definite concentration, above which increase in concentration of the hydrogen donator appears to have little or no effect on reduction velocity. At first sight this phenomenon might be explained on the usual grounds by assuming that the enzyme becomes "saturated" with regard to the donator, so that increase in concentration of substrate above this saturation point does not produce greater reaction velocity. There are, however, difficulties in the way of accepting this interpretation. For example, in some cases, *e.g.* propionic acid (Fig. 1) and succinic acid (Fig. 2), such a "critical" concentration is hardly apparent, and the curves do not exhibit the discontinuity which would be expected if the enzyme surface became truly saturated at some particular concentration of the substrate. In a number of cases a definite relationship appears to hold between the velocity of reduction and the concentration of the hydrogen donator. This is

$$v = k + k' \log c,$$

where v = velocity of reduction (reciprocal of time of reduction),
c = concentration of the hydrogen donator,
and k and k' are constants.

Beyond stating that in a number of cases we have found such a logarithmic relationship to accord with the experimental results, we do not wish to pursue the subject further at present, and hope to deal with it in detail in a later communication.

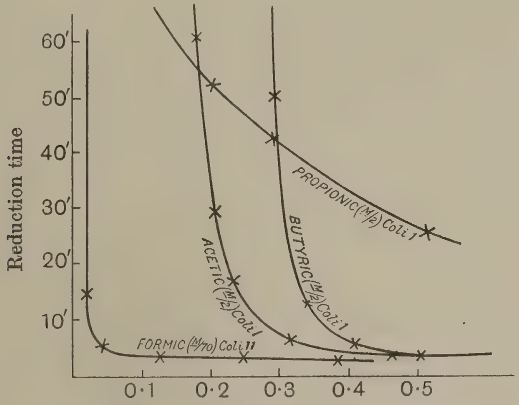

Fig. 1. Concentration of fatty acid

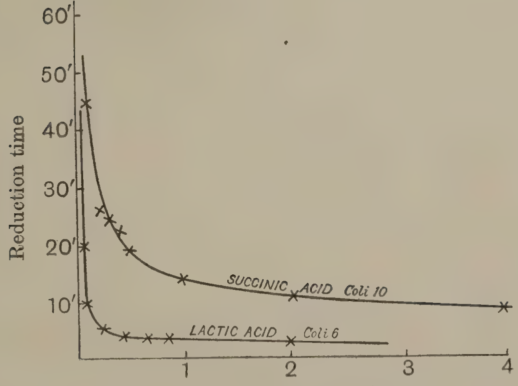

Fig. 2. Concentration (× $M/140$)

The products of the anaerobic oxidation of the fatty acids in presence of resting *B. coli* have not yet been investigated, but it is clear that in the case of formic acid there is a complete oxidation to carbon dioxide. There is a great increase in p_H during the anaerobic oxidation of formic acid, and carbon dioxide is evolved on treatment with acid.

Pakes and Jollyman [1901] have shown that *B. coli comm.* will decompose formic acid into equal volumes of carbon dioxide and hydrogen. If nitrates are also present in the nutrient medium, nitrites but not hydrogen are produced.

There is some evidence that the fatty acids have a retarding effect on reduction velocity at high concentrations, possibly due to adsorption of the acid on the organism. For instance, isobutyric acid has an optimum concentration as a hydrogen donator, being less active at higher concentrations. The same applies to isovaleric and caproic acids (see Table III).

Table III.

Conditions as in Table I.

Fatty acid	X cc.	Organism	Concentration of fatty acid	Reduction time in minutes
Isobutyric $M/20$	1·0	0·5 *coli* 1	$M/130$	No reduction
,, $M/2$	2·0	,,	$M/6{\cdot}5$	145
,, $M/2$	4·0	,,	$M/3{\cdot}25$	Not completely reduced in 6 hours
Isovaleric $M/2$	0·5	0·5 *coli* 3	$M/26$	57
,, $M/2$	1·0	,,	$M/13$	49
,, $M/2$	2·0	,,	$M/6{\cdot}5$	Not completely reduced in 2 hours
Caproic $M/2$	1·0	0·5 *coli* 4	$M/13$	74
,, $M/2$	2·0	,,	$M/6{\cdot}5$	Not reduced in 2 hours
,, $M/2$	3·0	,,	$M/4{\cdot}3$	,, ,,

It is possible that in the case of the higher homologues of caproic acid the reducing activity is masked by greater adsorptive effects which increase with the length of the fatty chain.

The saturated dibasic acids.

Oxalic acid is inert as a hydrogen donator, and malonic, glutaric, and adipic acids may also be regarded as inert, for under the conditions of our experiments these substances have never been found to bring about a complete reduction of methylene blue though they give indications of a slight activity (see Table IV).

Table IV.

Conditions as in Table I.

Acid	X cc. of neutralised acid sol.	Concentration of the acid	Reduction time in minutes
Oxalic $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	,,
Malonic $M/20$	1	$M/130$	,,
,, $M/2$	3	$M/4{\cdot}3$	Partial reduction not complete in 4 hours
Succinic $M/20$	1	$M/130$	11·0
,, $M/2$	3	$M/4{\cdot}3$	4·0
Glutaric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	Partial reduction not complete in 4 hours
Adipic $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	Partial reduction not complete in 4 hours

Oxalic, glutaric, and adipic acids (when mixed with succinic acid) do not retard the reduction due to the succinic acid, but malonic acid has a definite retarding effect. It is difficult to explain the anomalous behaviour of malonic acid, but there is no doubt as to the reality of the effect (see Table V).

Table V.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, 1 cc. of sodium succinate solution $M/20$, 1 cc. of the neutralised dibasic acid ($M/2$), and 1 cc. of water.

Acid	Reduction time in minutes
None (control)	11·0
Malonic	34·5
Glutaric	10·5
Adipic	10·2

The hydroxy acids.

Glycollic acid donates hydrogen only at relatively high concentrations, its behaviour being similar to that of acetic acid. Lactic acid, on the other hand, is an extremely powerful hydrogen donator even at low concentrations. Glyceric acid is active at lower concentrations than glycollic acid but is not as powerful as succinic acid. Tartaric acid is a very feeble donator, whilst citric acid is almost inert (see Table VI).

Table VI.

Conditions as before. *Coli* 5.

Acid	X cc. of neutralised acid sol.	Concentration of acid	Reduction time in minutes
Glycollic $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	3·0
Lactic $M/20$	1	$M/130$	3·0
,, $M/2$	3	$M/4{\cdot}3$	1·5
Glyceric $M/20$	1	$M/130$	45·0
,, $M/2$	3	$M/4{\cdot}3$	3·5
Tartaric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	21·0
Citric $M/20$	1	$M/130$	No reduction
,, $M/2$	3	$M/4{\cdot}3$	Not completely reduced in 1 hour

Fig. 3 gives the reduction time-concentration curves for glyceric and glycollic acids (in presence of 0·5 cc. of *coli* 5) and Fig. 2 for lactic acid (in presence of 0·5 cc. of *coli* 6).

The powerful activity of lactic acid compared with the other α-hydroxy acids is very striking. The product of anaerobic oxidation of lactic acid is pyruvic acid; the oxidation of lactic acid in presence of resting bacteria has been fully discussed in a previous communication [Quastel, Stephenson and Whetham, 1925]. The products of anaerobic oxidation of glycollic and glyceric acids have not yet been investigated.

The polyhydric alcohols.

Glycol donates hydrogen only at high concentrations. Glycerol is less active than glycol. Erythritol has no reducing effect (see Table VII).

Table VII.

Conditions as before. *Coli* 5.

Alcohol	X cc. of the $M/2$ alcohol solution	Concentration of alcohol	Reduction time in minutes
Glycol	0·5	$M/26$	Not reduced in 2 hours
,,	1·0	$M/13$	71
,,	3·0	$M/4{\cdot}3$	8·25
Glycerol	1·0	$M/13$	53
,,	3·0	$M/4{\cdot}3$	30
Erythritol	1·0	$M/13$	No reduction
,,	3·0	$M/4{\cdot}3$	,,

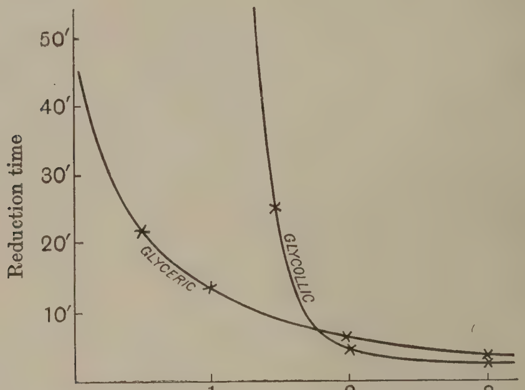

Fig. 3. Concentration of hydroxy acid (× $M/13$)

Glycol, glycerol and erythritol appear to have no retarding effect on reductions due to other substances. The penta- and hexa-hydric alcohols will be discussed later in connection with the sugars.

The glycerophosphoric acids.

α-Glycerophosphoric acid is a much more vigorous donator of hydrogen in presence of resting *B. coli* than glycerol itself, whilst β-glycerophosphoric acid is about as active as glycerol (see Table VIII).

Table VIII.

Conditions as before. *Coli* 5.

Hydrogen donator	X cc. of the $M/2$ H donator solution	Concentration hydrogen donator	Reduction time in minutes
Glycerol	1	$M/13$	53·0
,,	3	$M/4{\cdot}3$	30·0
α-Glycerophosphoric acid	1	$M/13$	2·5
,,	3	$M/4{\cdot}3$	2·0
β-Glycerophosphoric acid	1	$M/13$	70·0
,,	3	$M/4{\cdot}3$	20·0

The monohydric alcohols.

The members of this series from methyl to octyl alcohol have been investigated. From butyl alcohol upwards they do not appear to donate hydrogen and all inhibit or retard reductions by other substances. Since these higher alcohols are immiscible with water at ordinary temperatures, they do not lend themselves to quantitative investigation.

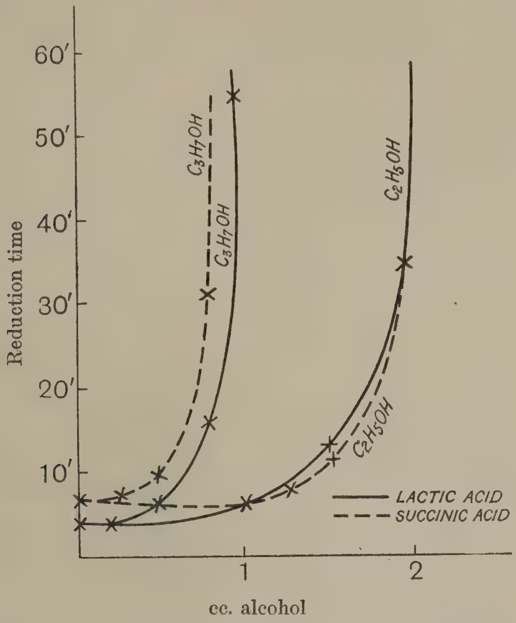

Fig. 4. Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. $M/20$ lactic acid and 1 cc. $M/20$ succinic acid.

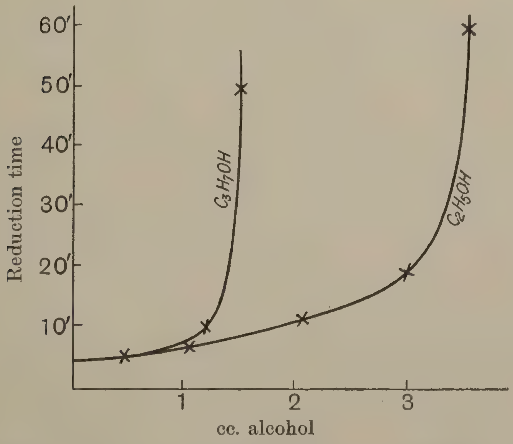

Fig. 5 Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. $M/20$ formic acid.

The lowest members—methyl, ethyl and propyl alcohols—all possess the characteristic property of inhibiting the reducing action of hydrogen donators. Total inhibition occurs at definite or critical concentrations of the alcohols, and these concentrations vary with the nature of the hydrogen donator (see Figs. 4, 5, which show the action of varying quantities of the alcohols on the reduction times of a definite quantity of donator, in the presence of 1 cc. of *coli* 7). The critical concentrations of alcohol vary very slightly with the quantity of resting organism used (see Fig. 6) and are independent of the concentration of the donator (see Table IX).

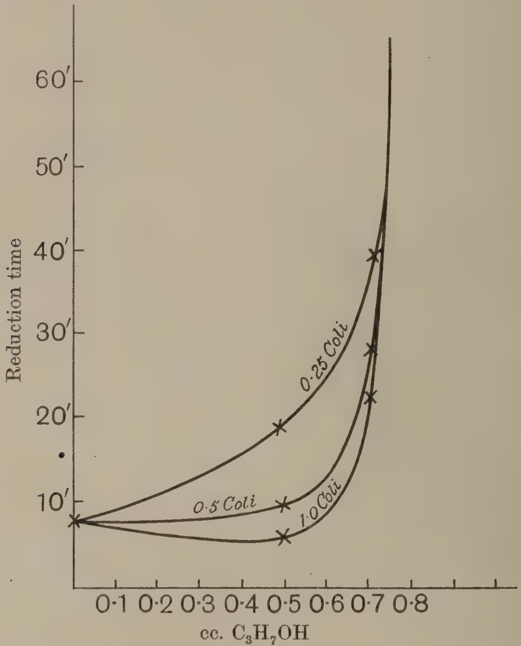

Fig. 6. Effect of propyl alcohol on reduction times of 1 cc. 1/5000 methylene blue by succinic acid.

Table IX.

Each vacuum tube contained 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, X cc. of 1 % sodium succinate, 0·7 cc. of propyl alcohol and 1 cc. of *coli* 7. The volume was made up to 7 cc. with water.

X cc.	Reduction time
0·5	Not reduced
1·0	„
2·0	„
3·0	„

These critical concentrations are least with propyl alcohol and greatest with methyl alcohol, *i.e.* methyl alcohol inhibits least vigorously.

Besides their property of inhibition, ethyl and propyl alcohols possess the power of donating hydrogen to methylene blue (see Table X). Methyl alcohol does not act as a hydrogen donator.

Table X.

Each vacuum tube contained 2 cc. of *coli* 8*, 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution and X cc. of the absolute alcohol. The volume was made up to 7 cc. with water.

Alcohol	X cc.	Reduction time in minutes
None (control)	—	23·25
Methyl	0·05	23·0
,,	0·1	23·0
,,	0·5	30·0
Ethyl	0·05	11·0
,,	0·10	10·4
,,	1·5	Not reduced
Propyl	0·05	10·25
,,	0·10	9·75
,,	0·50	10·9
,,	1·00	Not reduced

* *Coli* 8 was a highly reducing organism and is included here to demonstrate the alcohol inhibitions.

The effect of increasing the concentration of alcohol is to produce at first an increasing velocity of reduction. This reaches a maximum and remains constant until a certain concentration of alcohol is reached, when the velocity falls off sharply to zero.

Experiments with the vacuum U-tube (see previous papers) show that the inhibitant effects of the alcohols are not due to oxidation of leuco-methylene blue (as in the case of fumaric acid) but to some physical process, such as adsorption at the surface of the organism. The degree of inhibition is associated with the length and nature of the fatty chain: as shown above, high concentrations of some of the higher fatty acids also bring about a retardation of reducing velocity. The polyhydric alcohols, on the other hand, have no retarding effect.

Inhibitants.

An examination of the inhibiting action of substances other than the monohydric alcohols showed that:

1. Small quantities of cyclohexanol or cyclohexene completely inhibit all reductions by hydrogen donators.
2. Benzene, toluene, phenol, and acetone have definite but relatively slight inhibitory effects (see Table XI).

Table XI.

Each vacuum tube contained 1 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue, 0·2 cc. of 5 % sodium succinate, 0·5 cc. *coli* 9, and X cc. of inhibitant. The total volume was made up to 6·5 cc. with water.

Inhibitant	X cc.	Reduction time in minutes
Control	—	8
Benzene	0·5	9
Toluene	0·5	14
Phenol (10 % neutral)	0·5	10
Cyclohexanol	0·5	Not reduced in 6 hours
,,	0·2	,,
Cyclohexene	0·5	,,
Acetone	0·5	10

Reducing coefficient.

It is desirable to provide some means for the quantitative comparison of the reducing capacity of the large number of substances which can donate hydrogen to methylene blue in presence of resting *B. coli.* This may be done by comparing one donator with a standard donator—say succinic acid—under similar conditions. The most satisfactory method of comparison is to determine what concentration of a donator will reduce a given quantity of methylene blue in the same time as a definite quantity of the standard donator.

We propose, therefore, to define the *reducing coefficient* of a hydrogen donator with respect to a certain organism as the reciprocal of that (molar) concentration which will reduce 1 cc. of 1/5000 methylene blue solution in presence of a standard amount of the organism in half an hour, succinic acid being taken as a standard and its reducing coefficient being taken as 100. For the determination of the coefficient an amount of freshly prepared organism is taken which will bring about the reduction of 1 cc. 1/5000 methylene blue at p_H 7·2 and 45° in the presence of $M/700$ succinic acid in half an hour.

Table XII gives the reducing coefficients of the substances discussed in this communication. Ethyl and propyl alcohols have not been included, owing to their inhibitory action. The coefficients must only be regarded as approximate and as expressing the correct order of magnitude.

Table XII.

Hydrogen donator	Reducing coefficient (with respect to *B. coli comm.*)
Formic acid	700
Acetic acid	0·95
Propionic acid	0·46
Butyric acid	0·64
Valeric acid	0·5
Caproic acid	Less than 0·4
Caprylic acid	,, 0·4
Heptylic acid	,, 0·4
Nonylic acid	,, 0·4
Oxalic acid	,, 0·4
Malonic acid	,, 0·4
Succinic acid...	100
Glutaric acid	Less than 0·4
Adipic acid	,, 0·4
Glycollic acid	0·93
Lactic acid	583
Glyceric acid	5·2
Tartaric acid	0·46
Citric acid	Less than 0·4
Glycol	0·86
Glycerol	0·46
Erythritol	Less than 0·4
α-Glycerophosphoric acid	4·31
β-Glycerophosphoric acid	0·4

SUMMARY.

An account is given of the behaviour of a number of substances as hydrogen donators in presence of resting *B. coli* as the activating source and of methylene blue as the hydrogen acceptor. The following groups of substances are dealt with:

1. Fatty acids.
2. Saturated dibasic acids.
3. Hydroxy acids.
4. Polyhydric alcohols.
5. Monohydric alcohols.

The inhibiting action of the monohydric alcohols and of other substances is discussed. A table is given of the relative reducing powers or reducing coefficients of the hydrogen donators.

We wish to express our indebtedness to Sir F. G. Hopkins for the interest he has taken in this work and the thanks of one of us (M. D. W.) are due to the Medical Research Council for a grant held during this work.

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ERRATA

Vol. xix, pp. 464 and 474. The drop volumes in these papers are expressed in thousandths of a cubic millimetre ($\text{mm.}^3 \times 10^{-3}$) and *not* in mm.^3 as stated in the text.

LXXVIII. THE SOLUBILITY OF PROTEINS AND PROTEOSES IN ALDEHYDES AND OTHER ORGANIC SOLVENTS.

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(*Received May 12th, 1925.*)

Although the action of aqueous solutions of aldehydes upon proteins has been extensively studied, the behaviour of aldehydes in the anhydrous state does not seem to have been investigated in detail, except for the single observation of Mathewson [1906] that gliadin appeared to be somewhat soluble in impure benzaldehyde. Apart from its chemical interest such an investigation might lead to improved methods of separation and purification of proteins and proteoses. The present methods of fractionation depend largely on the use of inorganic salts, and there is a considerable field for the application of organic solvents in protein chemistry.

Observations were made in the first place with benzaldehyde. It was found that, whilst gelatin, egg-albumin, gliadin, and proteoses (Witte's peptone) were precipitated from their solutions in anhydrous phenol by paraldehyde, the addition of benzaldehyde had no effect. Egg-albumin was, furthermore, slightly soluble in benzaldehyde alone, and was reprecipitated on the addition of alcohol. Gelatin, gliadin and caseinogen, however, appeared to be insoluble in benzaldehyde. Glycine and alanine were similarly insoluble, but proteoses dissolved in benzaldehyde with great readiness at ordinary room temperature, only a small residue being left undissolved. The proteoses were precipitated by the addition of various organic solvents (alcohol, acetone, chloroform, paraldehyde, mesityl oxide), or even by warming the benzaldehyde alone to 37°; on cooling the proteoses re-dissolved.

Benzaldehyde is thus characteristic (1) in exerting a selective solvent action upon intermediate hydrolytic products of proteins, amino-acids and proteins themselves being almost insoluble; (2) in being efficacious as a solvent for proteoses within only a very restricted range of temperature (approx. room temperature).

Similar experiments were carried out with many other aldehydes and derivatives, and the results are as follows:

Table I. *Solubility of proteoses.*

m-Toluylaldehyde	Sol. at room temp.	Insol. at 37°
Benzaldehyde cyanohydrin	,,	,,
Salicylaldehyde	Slightly sol. at room temp.	Readily sol. at 37°
Anisaldehyde	,, ,,	,, ,,
Acetaldehyde	Insol.	
Oenanthol	,,	
Chloral	,,	
Chloral hydrate	Sol. just above M.P. (57°)	
Butylchloral hydrate	,, ,,	
Chloral alcoholate	Insol.	
Phenylacetaldehyde	,,	
Cinnamaldehyde	,,	
Citral	,,	
Furfuraldehyde	,,	
Acetone	,,	
Mesityl oxide	,,	
Acetophenone	Sol. just above M.P. (20°)	Insol. on warming
Benzophenone	Insol.	

A consideration of these results shows that proteoses are only soluble in aldehydes of the aromatic type, *e.g.* compounds in which the —CHO group is directly attached to the benzene nucleus.

In other aldehydes, *e.g.* aliphatic and heterocyclic, the proteoses are insoluble.

Substitution of groups in the benzene nucleus of the aromatic aldehydes, although not unfavourable to the solubility of proteoses, sometimes had the interesting result of reversing the effect of temperature (cf. salicylaldehyde). It is noteworthy that proteoses were also soluble in acetophenone, but not in acetone or benzophenone.

It is quite possible that this apparent solubility is in reality determined by chemical factors, as previous treatment of proteoses with formaldehyde and nitrous acid destroyed their solubility in benzaldehyde. However, even in the case of aldehydes which exert no solvent action, chemical reactions apparently take place, *e.g.* proteoses and gelatin become yellow when warmed with phenylacetaldehyde.

Whilst proteoses were insoluble in chloral, they were soluble in fused chloral hydrate and butylchloral hydrate, but not in chloral alcoholate, in which one of the OH groups is replaced by an ethoxy group. In addition to proteoses, gelatin, albumin, gliadin, and lecithin were also soluble in chloral hydrate, and these substances were precipitated therefrom by paraldehyde, but not by alcohol. It is remarkable that the addition of the elements of water to chloral, forming a dihydric alcohol, in which the two OH groups are attached to the same carbon atom, should lead to the production of solvent action upon the foregoing colloids, as alcohol, glycerol, and chlorohydrin are without such solvent power.

The effect of adding various aldehydes to an ethereal solution of lecithin has also been studied. In certain cases precipitation of the lipin took place, but with some aldehydes the solution remained clear, indicating that in such aldehydes the lecithin was soluble. The results in general are similar to those obtained with the proteoses, but a survey of the following table will indicate a few exceptions.

Table II. *Ethereal solution of lecithin.*

Substance added	Observation
Benzaldehyde	No effect
m-Toluylaldehyde	,,
Salicylaldehyde	,,
Anisaldehyde	Ppt.
Acetophenone	,,
Acetaldehyde	,,
Paraldehyde	,,
Phenylacetaldehyde	,,
Cinnamaldehyde	,,
Oenanthol	No effect

The application of aldehydes as fractionating solvents may thus be serviceable in the separation and purification of proteoses and lipins, as was suggested in the case of paraldehyde [Cooper, 1924].

The influence of the factor of chemical structure has also been considered in connection with the action of aldehydes and ketones on amino-acids.

The action of formaldehyde on amino-acids is well known, and is, of course, the basis of Sörensen's method. Using the same technique, the action of formaldehyde, acetaldehyde, and acetone on amino-acids has been compared quantitatively. It was found that acetaldehyde reacted much less readily than formaldehyde with glycine and alanine, whilst in the case of acetone there was practically no chemical action at all. It was therefore of interest to study the distribution of acetone between water and certain proteins, especially as acetone has been employed as a protein precipitant [Piettre and Vila, 1920]. In fact, acetone has quite a general precipitating action on various colloidal solutions, *e.g.* proteins, starch, glycogen, dextrin, lipins.

The following tables give the quantitative results of experiments on the distribution of (*a*) acetone between water and gelatin, (*b*) acetone between water and egg-albumin (heat coagulum) at room temperature.

The estimation was carried out according to Messinger's iodometric method. Preliminary experiments showed that equilibrium was attained within 7 days.

Table III.

(*a*) *Gelatin.*

	Acetone concentration in water phase	Distribution coeff. $\frac{\text{(amount of acetone in 1 g. protein)}}{\text{(amount of acetone in 1 g. water)}}$
(I)	0·2 %	0
	2·0	2
	8·0	0
(II)	0·25	0
	0·50	0
	1·00	0
	2·00	0
	4·00	1·7
	8·00	1·2
	20·00	0

(*b*) *Egg-albumin.* (*Heat-coagulum.*)

2 %	2·5
8	0·0

A point of interest, difficult to understand, is the phenomenon of a maximum shown with respect to the distribution coefficient; below and above a certain critical range of acetone concentration there is no evidence at all of any absorption of acetone by the protein. Although these results differ from the observations made on the distribution of phenol between water and proteins, in which case the partition-law was found to hold [Cooper, 1912], there is very little evidence that acetone reacts chemically with amino-acids or proteins.

Accordingly, as in the case of alcohol, the action of acetone as a protein-precipitant is probably physico-chemical.

The fact that the behaviour of aldehydes towards proteoses depends so much upon chemical structure and temperature rendered it of great interest to investigate the influence of these factors in the case of other solvents.

It is well known that proteins are soluble in molten phenol and cresols, and the phenols therefore afforded a convenient group in which to study the effect of structural differences. Observations have also been made in the case of certain amines.

The tests were carried out with proteoses, gelatin, and egg-albumin at 40° (except when the substance had a M.P. above 40° in which case the experiment was carried out at a temperature just above the M.P.), with the following organic substances.

Phenol and derivatives.

Phenol, cresols, carvacrol, *p*-chlorophenol, *o*-nitrophenol, α-naphthol, hexahydrophenol, anisol, guaiacol, salol, methyl and amyl salicylates, benzyl alcohol.

The main results of the observations are as follows:

1. The solvent action of the phenols diminishes as the homologous series is progressively ascended.

2. The introduction of a chlorine atom into phenol diminishes solvent power, but to a less degree than a methyl group.

3. Solvent action is diminished by introduction of the $—COOCH_3$ group, and abolished by that of $—COOC_5H_{11}$, $—COOC_6H_5$, nitro and methoxy groups; and by hydrogenation of the benzene nucleus (hexahydrophenol).

4. α-Naphthol and benzyl alcohol have no solvent action on proteoses and gelatin, although Mathewson [1906] found that gliadin was soluble in benzyl alcohol.

Amines and derivatives.

The observations were made at room temperature (whenever possible) and at 40°, unless a higher temperature was necessary to melt the substance.

Amines and their derivatives belonging to the aliphatic, aromatic and heterocyclic types were employed. The following results were obtained:

(1) Aniline, *o*-toluidine, *m*-xylidine, methylaniline, chloroaniline, anisidine and phenylhydrazine dissolved proteoses on warming but not at room temperature.

(2) Dimethylaniline and ethylaniline, however, dissolved proteoses at room temperature, but not on warming, showing that the introduction of two methyl groups or one ethyl into the $—NH_2$ radicle had the peculiar result of reversing the temperature effect (cf. benzaldehyde and salicylaldehyde).

(3) Proteoses were slightly soluble in formanilide (46°), but insoluble in benzylamine, dichloroaniline, phenylethylamine, diphenylamine, α-naphthylamine, azobenzene, urethane, pyridine, and camphylamine.

Proteoses are thus insoluble in amines of the aliphatic and heterocyclic types, as was also observed in the case of the aldehydes.

SUMMARY.

The results as a whole indicate that the solubility of proteins, proteoses, and lipins in organic substances, *e.g.* aldehydes, phenols, amines, is readily influenced by the chemical structure of the solvent and by temperature, and such solvents may thus be of service in biochemistry for the selective extraction or purification of the colloidal constituents of protoplasm.

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LXXIX. THE GLUCOSE CONTENT OF NORMAL URINE.

By GUY SEFTON LUND and CHARLES GEORGE LEWIS WOLF[1].

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(*Received May 16th, 1925.*)

There has been a long-standing impression that normal urine contains a small but definite amount of glucose. This idea has recently received substantiation from the results of Benedict and Osterberg [1921], who undertook to estimate the amount actually present. This was found to be within the limits given in the article of Schulz [1910]. Similar amounts to those obtained by Benedict and Osterberg were also found by Sumner [1924], using an entirely different method. Sumner stated that about 60 % of the total reduction was due to sugar.

The above results have not remained uncriticised by other investigators. Greenwald, Gross and Samet [1924] who give a résumé of the history of the problem came to the conclusion that the sugars of normal urine consist of difficultly assimilable carbohydrates or of reducing substances derived from protein.

Höst [1923] attempted to gain information on this point by preparing osazones from normal urine. He was able to show that aqueous solutions containing 0·0025 % of glucose yielded typical crystals of glucosazone and urine containing 0·01 % of added glucose gave characteristic crystals. By the same procedure crystals were obtained in normal urine, but their appearance was sufficient to differentiate them from the glucose compound. The difference between the physiological carbohydrate and glucose added to the urine was also shown in the rapidity of the fermentation with yeast. The physiological carbohydrate was only completely fermented after 48 hours at 37°. It is thus seen that neither Greenwald and his co-workers nor Höst believe that glucose is a constituent of normal urine.

Determinations of the reducing power and optical rotation of urine before and after fermentation have been made, but as Greenwald, Gross and Samet point out, no one has ever demonstrated the production of either alcohol or carbon dioxide in the process.

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[1] Assisted by a grant from the Medical Research Council.

During the course of a study of the fermentable carbohydrates of the blood we were led to examine the action of yeast on normal urine. For this purpose we used the larger differential manometer of Barcroft [1914] with mechanical shaking.

1 cc. of urine, which had been previously well shaken to expel dissolved carbon dioxide, was mixed with 0·5 cc. of a 1 % orthophosphoric acid solution. This was fermented with 0·2 cc. of a thick suspension of yeast placed in the smaller cup of the apparatus. The control flask contained 1·0 cc. of water and the same amount of yeast and phosphoric acid as in the reaction flask. The yeast used was the strain employed by Grafe and Sorgenfrei [1924] in their studies of the fermentable sugar of the blood and was the powerfully fermenting "Rasse XII" of the Institut für Gärungsgewerbe in Berlin. It was transferred each day to sterile beer wort and the 48-hour growth centrifuged and washed three times with tepid water. It was then suspended in saline. The fermentations were usually continued for 60 to 90 minutes at 30°. When glucose was added to normal urine the rise in the manometer was usually over in 45 minutes. Many of the tests were performed in quadruplicate and the concordance was very satisfactory. Using the constants of the manometers as obtained by the method of Münzer and Neumann [1917] the carbon dioxide yielded by the glucose on fermentation accounted for about 85 % of the sugar used.

In more than a dozen samples of normal urine we have never obtained a positive pressure in the manometer. Normal urines to which glucose (equivalent to a concentration of 0·05 %) had been added showed a rise within 10 minutes after addition of the yeast, the total rise being of the order of 30 mm.

That glucose excreted by the kidneys in very small amount is detected by yeast is shown in the following experiment where 50 g. of glucose were administered to a woman, aged 51, suffering from a severe form of Graves' disease, with a B.M.R. of + 80 %. The results are shown in the following table which is one of three practically similar experiments on the same patient.

Time	Blood sugar %	Sugar in urine (Benedict)	Rise in manometer mm.
9.00	0·088	0	0
10.30	0·256	±	131
11.30	0·120	0	0

50 g. of glucose at 9·05.

The amount of glucose excreted in the second sample of urine was barely enough to be detected with Benedict's reagent, and we were unable to detect any rotation with a small polarimeter reading to 0·1° and using a 200 mm. tube.

SUMMARY.

Using the differential manometer of Barcroft no carbon dioxide production has been found when normal urine is treated with yeast. This points to the absence of glucose from normal urine.

In the present form the manometric method appears to be among the most sensitive and selective means which we possess for detecting glucose in the urine.

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LXXX. ESTIMATION OF SUGAR IN SMALL QUANTITIES OF BLOOD.

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(*Received May 16th, 1925.*)

The method is a modification of the one described by Shaffer and Hartmann [1921]. It has been in use since 1921 and has found its way into several laboratories in Holland.

Solutions

1.	Phosphotungstic acid[1]	5 g.
	Sodium sulphate (pro analysen) ...	20
	Sulphuric acid (pure)	2
	Water to	1000 cc.
2.	Copper sulphate (crystals)	1·5 g.
	Tartaric acid	3·75
	Sodium carbonate (anhyd.)	40·0
	Potassium iodide	2·0
	Potassium iodate	0·2
	Water to	1000 cc.

Procedure. The blood is mixed with 25 volumes of solution 1. For one determination and a blood-sugar content between 0·07 and 0·20 % one can take conveniently 6·8 cc. of solution 1 and add 0·2 cc. of blood. When one expects more than 0·2 % of blood-sugar one should take 50 of solution 1. In rare cases the blood is to be diluted still more. When the blood-sugar is lower than 0·07 % the blood should be diluted with only 10 volumes of solution 1 and more blood should be taken. For double determinations the amounts of blood and solution 1 should be doubled. After thorough mixing the haemoglobin begins to settle to the bottom as haematin within a few minutes. It then gives a clear and colourless filtrate when filtered through paper. The paper must be starch-free and should not give off any fibres to the filtrate.

5 cc. of the filtrate are carefully pipetted into a large test-tube of 23 mm. diameter; 5 cc. of solution 2 are added and the tube is covered with a well-fitting glass cover in the form of a small crystallising dish. The test-tube is

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[1] I always used a specimen from Merck.

placed in a boiling water-bath for 15 minutes. I had special baths made for the purpose in which six tubes could be heated at the same time (care should be taken that there is room to move the burner freely to and fro underneath the bath without lifting the bath itself).

Exactly 15 minutes after the beginning of the heating the tube is cooled in water. 5 cc. of *N* sulphuric acid are now added and mixed well with the contents; the tube is again covered and shaken at intervals during 2 minutes, after which time the liberated iodine is titrated with thiosulphate (0·08 *N* is a convenient strength; it deteriorates less than the weaker solutions, but it should be kept in a microburette).

For calculation of the amount of reducing substance in the form of glucose the amount of copper reduced is calculated from the formula

$$W = D \times 63{\cdot}56,$$

where *W* is the amount of copper; *D* is the difference between the determination and the value of a blank (where instead of 5 cc. of filtrate 5 cc. of solution 1 are used) expressed in cc. of *N* thiosulphate; and 63·56 is the atomic weight of copper.

The amount of reducing substance as glucose is read from Table I.

Table I. (*Reduction table.*)

Copper mg.	Glucose mg.
0·050	0·090
0·100	0·116
0·150	0·142
0·200	0·168
0·250	0·195
0·300	0·221
0·350	0·247
0·400	0·273
0·450	0·300
0·500	0·327
0·550	0·358
0·600	0·395
0·650	0·447

Interpolation table to be used between 0·05 and 0·500 mg. of copper	
$\frac{0{\cdot}026}{50} \times 2 = 0{\cdot}001$	
4	02
6	03
8	04
10	05
12	06
14	07
16	08
18	09
20	10
22	11
24	13
26	14
28	15
30	16
32	17
34	18
36	19
38	20
40	21
42	22
44	23
46	24
48	25
50	26

In Table II are given values of blood-sugar found with the use of the method described in comparison with MacLean's method [1919], which was tested in this laboratory by Bahlmann [1920].

Table II. *Comparison with MacLean's method.*

	Mg. glucose per 100 cc. of blood	
	MacLean	Author's method
1	65	73
2	71	63
3	124	116
4	158	164
5	178	159
6	253	270
7	398	408
8	488	483

Wetselaar [1924] working with the method described obtained the following results (Table III) (reducing his values of 0·005 *N* thiosulphate to mg. of glucose by Table I):

Table III. *Values derived from Wetselaar's work.*

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Glucose taken mg.	Glucose found mg.
0·090	0·088
0·112	0·113
0·135	0·138
0·168	0·168
0·179	0·183
0·224	0·222
0·258	0·264
0·280	0·280
0·314	0·313
0·336	0·339
0·370	0·371
0·392	0·391
0·426	0·433
0·448	0·455

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LXXXI. HAEMOCYANIN. PART I.

THE DISSOCIATION CURVES OF THE OXYHAEMOCYANIN IN THE BLOOD OF SOME DECAPOD CRUSTACEA.

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(*Received May 18th, 1925.*)

The blue colour of the blood of certain arthropods and molluscs is due to the presence of a protein, containing copper, which exists in oxidised and reduced forms; the former, *oxyhaemocyanin*, has a blue colour, whilst the latter, *haemocyanin*, is colourless. The transformation of the oxidised into the reduced form is readily accomplished by exposing blood containing oxyhaemocyanin to a vacuum or by saturating it with an inert gas; the reverse change takes place spontaneously on again bringing the blood into contact with air. Oxyhaemocyanin has been isolated in crystalline condition from the blood of certain species. [For bibliography see Dhéré, 1915.]

The properties of haemocyanin immediately suggest that this pigment exercises respiratory functions analogous to those of haemoglobin. This view has, in fact, been almost generally accepted and has received support from determinations, by various authors, of the oxygen capacities of the bloods of a number of marine invertebrates. In the case of those species which contain haemocyanin, the values obtained, although differing widely from species to species and even from individual to individual of the same species, were considerably higher than the oxygen capacity of the sea-water in which the animals lived. With those species containing no haemocyanin, on the other hand, the values approximated to that of sea-water. This is illustrated by the following figures, which are quoted from Winterstein [1909].

Containing haemocyanin			Not containing haemocyanin		
Octopus	*Palinurus*	*Maia*	*Ascidia*	*Pinna*	*Patella*
4·2–5·0	1·48–1·43	1·13–0·84	0·48–0·38	0·57	0·68 vols. %

Alsberg and Clark [1914], however, state that the blood of *Limulus*, when exposed to a vacuum, yields an amount of oxygen which is not significantly greater than that obtained under the same conditions from sea-water. Whilst not denying that haemocyanin possesses the power of combining with oxygen, these authors conclude from their results that this pigment can only function as a carrier of oxygen by a mechanism other than that of the physical dissociation of oxyhaemocyanin. The same authors [1910] had previously concluded, on other grounds, that haemocyanin from *Limulus* differs from haemocyanin from *Octopus*.

It is evident that the proof that haemocyanin exercises respiratory functions in virtue of its power to dissociate would be greatly strengthened if it could be shown that the conditions which govern the equilibrium between the oxidised and reduced forms were adapted to the transport of oxygen within the organism. A comparative study with different species would, moreover, throw light on the question of the possible existence of different haemocyanins. The present investigation, which forms part of a more comprehensive one, was undertaken with this object and deals with the oxyhaemocyanin dissociation curves of the blood of the four decapods, *Homarus*, *Palinurus*, *Maia* and *Cancer*.

EXPERIMENTAL.

The determinations of the oxygen content of bloods containing haemocyanin which have hitherto been carried out have been made either by a titration method, involving the use of sodium hydrosulphite ($Na_2S_2O_4$), or by analysis of the gases removed from the blood by evacuation. Of these methods, the former is open to obvious objections and would, moreover, not be applicable to determinations at low oxygen tensions; the latter is capable of yielding extremely accurate results but requires a special mercury pump which was not available. It therefore appeared desirable to adopt one of the methods normally employed in the case of vertebrate blood. These depend upon the liberation of oxygen from oxyhaemoglobin by the use of potassium ferricyanide. A preliminary experiment carried out with the Barcroft differential apparatus indicated, however, that this reagent did not act similarly towards oxyhaemocyanin. In the absence of the discovery of a substitute for the ferricyanide, the use of the Barcroft or Haldane apparatus for the determination of the oxygen capacity of blood containing haemocyanin is thus impossible. On the other hand, the apparatus devised by Van Slyke and Neill [1924] combines the action of ferricyanide with vacuum extraction, and in its most recent modification [Harington and Van Slyke, 1924] permits the removal of the extracted blood from the apparatus under conditions which preclude recombination with the extracted gases. It therefore appeared that this latter modification would be applicable to the problem under discussion provided that extraction alone was sufficient to cause the complete dissociation of the oxyhaemocyanin contained in the blood. Trial experiments with this apparatus showed that extraction for 1–2 minutes with constant shaking in contact with mercury caused the complete disappearance of the blue colour. Moreover, determinations of the oxygen capacities of the bloods of *Maia* and *Palinurus*, after equilibration with air, gave values of 1·29 and 1·80 vols. % respectively, which are of the same order as those obtained by Winterstein and quoted above. These results were considered to demonstrate the applicability of the Van Slyke apparatus, which was accordingly employed for the measurements recorded in this paper.

The blood was collected by the following methods, for instruction in which the authors are indebted to Dr L. T. Hogben:

Homarus vulgaris (Common lobster). An incision was made between the terminal and adjoining terga of the abdominal segments and was accompanied by section of the abdominal artery. This caused a ready flow of blood which was collected and placed in the ice chest until the clotting process was complete. The freshly drawn blood was pale pink in colour but rapidly became purplish blue on exposure to air. The clotted blood was defibrinated by grinding with a small quantity of powdered glass and filtering through bolting silk.

Palinurus vulgaris (Rock lobster). An identical method was employed. The blood of this animal differed from that of *Homarus* in containing a greater amount of pink pigment and in clotting with much greater rapidity.

Maia squinado (Spider crab). The carapace was opened over the heart and an incision made in this organ. The outflowing blood, which contained only a trace of pink pigment, quickly became blue. Apart from agglutination of the leucocytes, which were removed by filtration, no clotting occurred.

Cancer pagurus (Common crab). The method employed was identical with that used in the case of *Maia*. The blood did not clot, was practically colourless when drawn, and assumed a rich blue colour, unmasked by pink pigments, when in contact with air.

The following technique was employed for the determinations. About 8 cc. of blood were drawn into a tonometer, equilibrated at room temperature (15°) with an analysed mixture of oxygen and nitrogen, and then transferred by displacement with mercury to an Ostwald pipette provided with a stop-cock. 5 cc. were introduced into the Van Slyke apparatus, two drops of octyl alcohol added to prevent foaming, and the blood extracted by shaking in the chamber for 3 minutes. The blood was then removed through the trap and the gas analysed by the method described by Harington and Van Slyke, the manometer readings being made at a volume of 0·5 cc. The method employed for equilibration was tested by equilibrating two samples of the same blood, one of which was in equilibrium with the air whilst the other had been freed from oxygen by evacuation, with a low oxygen tension. Analyses of the two samples yielded identical results, thus showing that the method permitted equilibrium to be reached reversibly.

All analyses were made within 12 hours of drawing the blood, which was stored in the ice chest when not in use. It was proved by determinations on the blood of *Maia* that, under these conditions, no loss in oxygen-combining power occurred during that period. When sufficient blood was required for a series of determinations, a uniform specimen was obtained by mixing the blood of two or more animals. The results were calculated according to Van Slyke's equation and are given in the following table:

Species	Number of animals	Oxygen tension (mm.)	Oxygen capacity of blood (vols. %)	Oxygen capacity of haemocyanin (vols. %)*	% saturation of haemocyanin
Maia	2	8·0	0·31	0·28	24·5
		19·8	1·05	0·94	82·5
		77·9	1·40	1·12	98·2
		149·8	1·75	1·22	100
		(347·0)†	(2·39)	1·14	—
		741·0	3·66	—	—
Palinurus	2	11·0	0·47	0·43	35·6
		20·8	1·13	1·05	86·0
		79·0	1·49	1·20	99·9
		148·6	1·80	1·25	100
		(344·1)	(2·49)	1·22	—
		735·0	3·88	—	—
Cancer	3	13·5	0·90	0·86	46·5
		21·7	1·69	1·63	88·1
		80·0	2·09	1·85	100
		149·8	2·29	1·85	—
		(289·0)	(2·74)	1·85	—
		741·0	4·02	—	—
Homarus	3	16·5	0·61	0·56	76·6
		22·5	0·84	0·76	—
		80·3	0·99	0·73	100
		147·8	1·22	0·73	—
		(342·5)	(1·88)	0·73	—
		732·5	3·22	—	—
Maia	3	Air	1·68	—	—
,,	3	,,	1·29	—	—
Homarus	1	,,	1·18	—	—
,,	1	,,	1·09	—	—
Cancer	1	,,	0·96	—	—

* Obtained from curves in Fig. 1.
† The figures in brackets were obtained by interpolation.

The results of the first four experiments have been used to plot the oxyhaemocyanin dissociation curves shown in Fig. 1. The method employed for this purpose is illustrated by Fig 2, which refers to the blood of *Cancer*. Curve *A* has been plotted from the experimental values and hence represents the relation between the oxygen capacity of the blood and the oxygen tension. *B* represents the physically dissolved oxygen and has been obtained by drawing a line through the origin parallel to the region of *A* in which the capacity is proportional to the oxygen tension. The differences between the ordinates of *A* and *B* give the amounts of oxygen bound to haemocyanin at any particular tension. These have been plotted in *C*, which thus represents the dissociation curve of the oxyhaemocyanin in the blood. *D*, the oxygen capacity curve for sea-water at the temperature of the experiments has been added for comparison. All the curves in Fig. 1 have been obtained from the experimental results in this manner.

DISCUSSION.

It is at once evident from the curves reproduced in Fig. 1 that not only does oxyhaemocyanin yield oxygen to low tensions of this gas but that it does so under conditions which render it capable of exercising real respiratory functions within the organism. Corresponding with the low oxygen tension of the medium in which the animals live, the haemocyanin is, under temperature conditions which approximate to the normal, about 85 % saturated when in equilibrium with oxygen tensions of 20 mm. At the lower tensions which doubtless exist in the tissues, dissociation would occur, thus providing the latter with oxygen. Haemocyanin may thus be regarded as a respiratory pigment which functions in a manner analogous to that of haemoglobin.

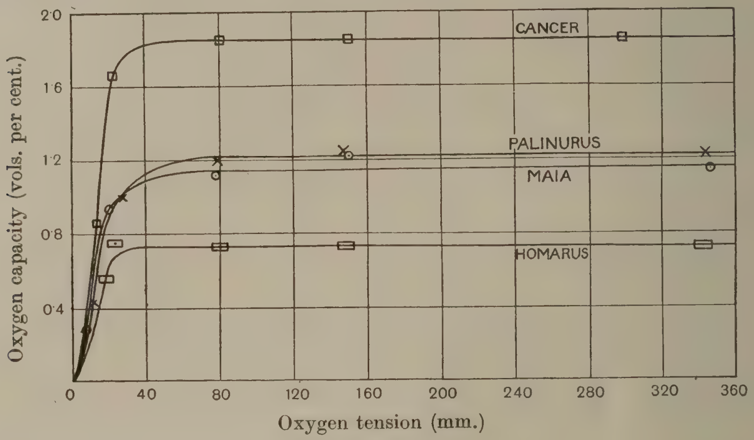

Fig. 1.

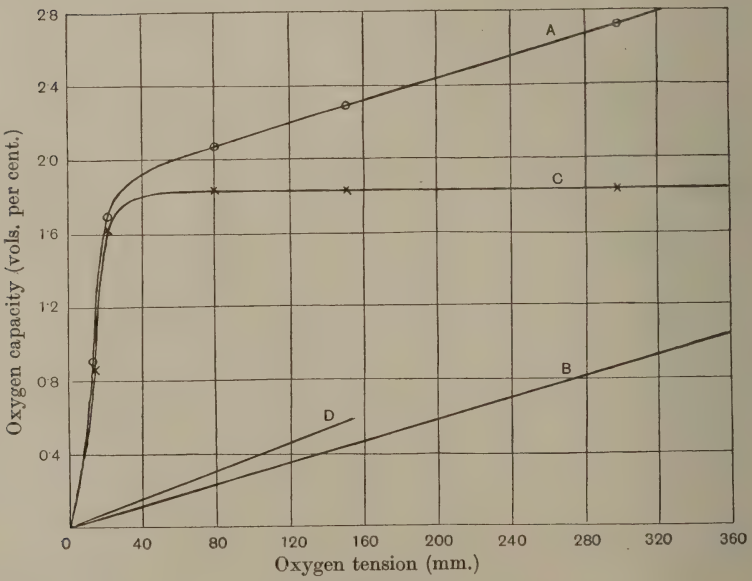

Fig. 2.

Whilst a comparison of these two proteins must be deferred until further experimental work has been carried out on the various conditions modifying the affinity of haemocyanin for oxygen, attention may be directed to the tendency of the curves in Fig. 1 to assume the S-shaped form characteristic of the curve for haemoglobin. Should this form of the curve be confirmed by further work, an interesting analogy between the two proteins will appear and experiments will be carried out to ascertain if it is dependent on the same factors which are responsible for the double inflexion in the haemoglobin curve.

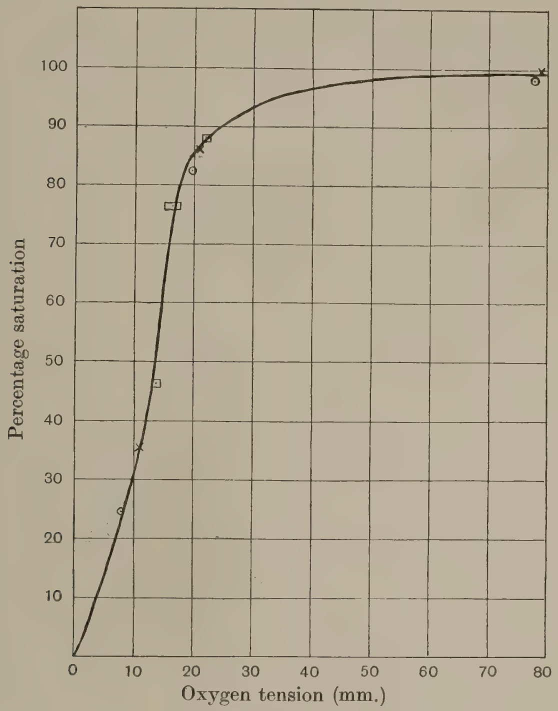

Fig. 3.

⊙ = Maia
⊡ = Cancer
× = Palinurus
▭ = Homarus

When a comparison is made between the oxyhaemocyanin dissociation curves for the four species examined it is found that whilst the oxygen capacities of the bloods differ widely in magnitude, the shapes of the curves show a close resemblance. If, further, the oxygen tension at which the haemocyanin is 50 % saturated is selected as a measure of its affinity for oxygen it is found, within the limits of experimental error, that identical values (14 mm.) are yielded by the four curves. The significance of these results becomes more evident when a composite curve, in which the percentage saturation is plotted against the oxygen tension, is drawn. This has been done in Fig. 3, omitting one reading for *Homarus* which is obviously erroneous (see Fig. 1) and also the higher oxygen tensions at which the haemocyanin is 100 % saturated. The points all lie on a smooth curve which retains, and in fact emphasises, the S-shaped form mentioned above. Whilst, in the absence of corroboration of a different nature, we should hesitate to assert the identity of the haemocyanins present in these species, these results, nevertheless, are very strong evidence in its favour.

In this connection, reference may be made to a paper by Redfield and Hurd [1925] which appeared while this work was in progress. These authors utilised the blue colour of the oxyhaemocyanin itself for the colorimetric estimation of the percentage saturation, at various oxygen tensions, of the haemocyanin present in the bloods of *Limulus* and *Loligo*. The shapes of the two dissociation curves obtained in this manner show little resemblance, but when the curve for *Limulus* is compared with those obtained in this investigation an unmistakable similarity is found to exist. This similarity extends, moreover, to the oxygen tensions at half saturation, the value for *Limulus* approximating to that given above. This corresponds not only with the fact that the animals are members of the same phylum, but also with the similarity in their habits and environment.

It will be observed that the oxygen capacities as determined by the Van Slyke apparatus are somewhat higher than those obtained by Winterstein using a mercury pump. This may be connected with the causes which are responsible for the well-known discrepancy between the results obtained with the Van Slyke and the Haldane apparatus in the case of vertebrate blood. Nevertheless it must be emphasised that considerable variations in the haemocyanin content of the blood of different individuals of the same species do actually occur. This is evident, not only from the values which are given in the table, but also from the differences in the intensities of the colour of the blood.

Curves *B* and *D* in Fig. 2 show that the amount of oxygen physically dissolved in the blood of *Cancer pagurus* is almost the same as that dissolved in sea-water when in equilibrium with the same oxygen tension. With the other three species the agreement was even closer. This accords with the observations of various workers [Höber, 1922] that the freezing points and consequently the salt concentrations of the blood of marine invertebrates are practically identical with those of sea-water.

SUMMARY.

1. The dissociation curves of the oxyhaemocyanin present in the blood of *Maia*, *Palinurus*, *Cancer* and *Homarus* have been plotted from determinations of the oxygen capacities of blood equilibrated with oxygen at various tensions.
2. The affinity of the haemocyanin for oxygen is the same for all four species.
3. The results show that haemocyanin is capable of exercising respiratory functions in a manner analogous to haemoglobin, and suggest that the haemocyanins present in the species examined are identical.

We should like to take this opportunity of thanking the Committee of the British Association for the use of tables at the Marine Biological Laboratory, and Dr E. J. Allen, F.R.S. and the Staff at Plymouth for their kind encouragement, and for the facilities which they placed at our disposal. The expenses of the research were, in part, met by a grant from the Moray Fund of Edinburgh University.

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LXXXII. THE CHANGES IN CONDUCTIVITY OF RED CELL SUSPENSIONS DURING HAEMOLYSIS.

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(*Received May 27th, 1925.*)

Since Stewart [1899] first described the changes occurring in suspensions of cells subjected to the action of lysins, numerous investigators have applied his methods, with various modifications, to the same and similar subjects. With the application of the methods to the changes occurring in suspensions of erythrocytes we are particularly concerned, for some of the findings vary from each other, as well as from the results obtained by other methods.

The available methods for studying conductivity are three in number—the Kohlrausch method, the rotating commutator method of MacGregor, and the method of the ballistic galvanometer. The last is only of use as a final resort in certain special cases, since the effects of polarisation are not excluded.

The Kohlrausch method as used by Stewart in his original researches is open to grave objection. In the first place, the use of heavily platinised electrodes is a disadvantage as well as a necessity, for considerable error may be introduced when solutions containing substances such as the serum proteins or saponin are employed, since they may be deposited on the electrodes, thereby altering the resistance. This difficulty is overcome by Brooks [1923] who uses blank electrodes, and obtains the necessary improvement in the telephone minimum by the insertion of a variometer in the bridge circuit, as was also done by Osterhout and others. The method suffers from the disadvantage that it is dependent on the observing of a minimum of sound produced by the exact position of a sliding contact, interpolations between the effects produced by two different positions of the slider being impossible.

The remaining method avoids these faults. The electrodes do not need to be platinised, though platinised electrodes which have been heated have an advantage over polished electrodes; provided the commutator is run at a fair speed, polarisation is as completely eliminated as in the Kohlrausch method, galvanometer readings replace the subjective determination of a sound-minimum, and interpolation to any desired degree of accuracy is possible.

With the selection of a suitable electrical apparatus for the measurement of the conductivities the difficulties do not end, for it is necessary that the cell-containing fluids shall be such as will lend themselves to accurate measurement. Stewart used defibrinated blood as the basis of his experiments, the cells being thus suspended in serum. This is not an ideal suspension medium for conductivity work; the cells are too numerous, rouleaux are present, sedimentation is rapid, the suspension is thick and difficult to remove from the electrodes, and the presence of the serum introduces fallacies, not only by the contained proteins inhibiting some forms of lysis, and by their tendency to be adsorbed on the electrodes, but because of their liability to spontaneous alterations of conductivity [Paoli, 1922]. Better results are obtained from suspensions of cells in saline, in which these fallacies are not encountered. The effect of any one of the above-mentioned factors in occasioning error may be small, but their added effects very great indeed.

Details of the apparatus employed, which was specially made to reduce these disturbing influences to a minimum, are given below.

The Rotating Commutator. One great advantage of the rotating commutator method is that, since the telephone is replaced by a galvanometer, the exact balancing resistance of the Wheatstone bridge can be interpolated from the deflections observed when resistances on either side of it are inserted. The galvanometer may be of any desired sensitiveness or the deflection for 1 ohm difference may be varied by alteration of the voltage. Moreover, the fact that a series of observations on both sides of the zero should give consistent results affords a valuable check.

The method was first described by MacGregor, was employed by Fitzgerald, and later by Whetham. The earlier forms of commutator were not very satisfactory in use, and several years ago one of us devised a pattern which obviated the defects. This model was shown at the Royal Society of Edinburgh, and at the International Congress of Physiology in Edinburgh, 1922. A brief description may be given. An ebonite cylinder of 10 cm. diameter and 3·5 cm. length is mounted on a ball-bearing axle. On the periphery are mounted a number of brass plates, moderately tapered, so that the gaps between them are wider at the one end of the cylinder. The drum now in use has 22 such plates, although for certain purposes the number should be a multiple of 4. After being fixed the plates are divided, giving in effect two coaxially mounted cylinders with the corresponding tapered plates all accurately oriented. Two concentric brass rings are let into each end of the cylinder, and the plates connected alternately to the outer and inner rings of the corresponding end. Contact with the plates and rings is made by brushes of fine brass wire or thin brass sheet. The brushes on the plates are placed so that when one is in contact with one plate, the other is in contact with a plate of the alternate series, so that, on rotation of the drum, a direct current applied to the rings is taken off by the plate brushes as an alternating current. This is led to the conductivity apparatus, from which it is taken back to the other end of the drum, where it is reconverted to a direct current and is led to the galvanometer. The end with the broader plates is used as the alternator, the object of the taper being to secure that the galvanometer contacts are made after, and broken before, the corresponding contacts on the alternator.

The Conductivity Cell. The cell used for these determinations was made for the special purpose in view. It consists of a glass tube of 6 mm. bore, the small platinum electrodes being placed vertically with their lower edges about 10 mm. from the bottom of the tube; with 1 cc. of liquid in the cell, the level of the liquid is 10 mm. above the tops of the electrodes. The part of the tube where the electrodes and their side tubes are situated is slightly blown out, so that a pipette can be passed to the bottom of the cell without contact with the electrodes. By these means, irregularities due to settling of suspensions on the electrodes and to sedimentation are reduced to a minimum. The electrodes should be grey, very slightly platinised.

Method. The conductivity cell is immersed in a thermostat, a large rectangular glass vessel electrically heated and controlled, the contents of which are stirred by a current of air. The temperature in these experiments was maintained at 25°.

In place of a bridge with a sliding contact, two resistance boxes are used. It is a convenience to keep the one box constant throughout and of the same resistance as the box in the cell circuit, so that the resistance of the cell, when balanced, is the same as that of the second box. The exact balancing resistance, to fractions of 1 ohm, is obtained by interpolations from the galvanometer deflections. The latter, of course, can be varied at will by altering the voltage applied.

The galvanometer employed is a Paul unipivot instrument of 732ω, with a centre zero.

One very necessary precaution is to be mentioned. When the liquid in the cell is changed to another of different conductivity, the electrodes must be soaked repeatedly—some three or four times—with fresh portions of the fluid. If this is not done, errors of a magnitude greater than the differences one requires to measure may be introduced, and failure to carry out this soaking may completely invalidate results.

RESULTS.

We propose to present the results as a series of illustrative experiments, representative of a large number. It may be well to point out at the beginning the object of the experiments. As a result of some of the earlier work, it is generally held that conductivity measurements of cell suspensions at various stages of lysis by various haemolysins show that the haemoglobin and the electrolyte of the red cell may be liberated independently of each other, and that, in some cases, the pigment may escape while the electrolyte is retained. Such results are used to support the idea that the haemoglobin and electrolyte are combined to a cell-stroma, the existence of which, however, is made doubtful by other researches. The accuracy of this early work is scarcely sufficient for conclusions of such importance to be based upon it; we have therefore repeated the fundamental experiments with the superior methods at our disposal, in order to find to what extent the usually accepted facts are correct, as well as to see if any alternative explanation of the observations can be offered.

1. *Effect of saponin on saline.*

		κ
Normal saline	...	0·01515
Plus saponin	...	0·01517

The amount of saponin added was about 5 mg.; the saponin used being Merck's purest. The effect on the conductivity of the saline is seen to be negligible.

2. *Effect of addition of water to cell suspension.*

The cell suspension used in this and other experiments was prepared by suspending the washed cells from 1 cc. of human blood in 5 cc. of normal saline (0·85 % NaCl). Such a suspension is of convenient strength, but it is to be noted that it contains fewer cells than the defibrinated blood used by Stewart in his work. Serum proteins are, of course, absent. The conductivity of this suspension was first taken alone, after which a known volume of water was added in order to produce laking of the cells. The conductivity was then again measured, and thereafter saponin (about 5 mg.) added to bring about complete lysis. Again the conductivity was ascertained:

	κ	
Normal saline	0·015180	
Plus 2 vols. H_2O	0·005572	
Suspension	0·013600	
Plus 2 vols. H_2O	0·005325	Lysis 51 %
Plus saponin	0·005648	

It is clear that as some of the cells haemolyse, electrolyte passes out of them along with the haemoglobin, for the diluted suspension, had no lysis occurred, should have given $\kappa = 0{\cdot}004991$. All the electrolyte does not escape from the cells of the suspension, as shown by the further increase of conductivity on the addition of saponin, which produces complete lysis; this is simply because only about half the cells were laked by the two volumes of water. On the other hand, the lysis of the first 50 % of the cells by the water gives very nearly the same increase of conductivity as does the lysis of the second 50 % by the saponin, and there appears no evidence that electrolyte is retained in the haemolysed cell, and that it is not liberated as freely as is the pigment by the action of the water. Stewart does not seem to have taken into account the fact that, on his additions of various amounts of water to the cells, only a certain percentage haemolysis must have resulted, and when he says that it is possible, by the addition of a large quantity of water to bring out of the corpuscles as great a quantity of electrolyte as by any other method of laking employed, *e.g.* saponin, it would appear that the facts might be equally correctly accounted for by saying that a sufficiently large quantity of water will lake as many cells as will saponin, and therefore liberate as much electrolyte. Stewart adds as much as six volumes of water to defibrinated blood—an amount which will produce complete lysis—but at the same time most of his results are for volumes of water which do not produce complete lysis; there is, however, no note of the amount of haemolysis in each case, nor is this taken into account.

Take this similar experiment:

	κ.	
Normal saline	0·015590	
Plus 1 vol. H_2O	0·008408	
Suspension	0·014434	
Plus 1 vol. H_2O	0·008019	Lysis 28 %
Plus saponin	0·008612	

The conductivity of the suspension diluted with one volume of water, had there been no lysis, may be calculated to be $\kappa = 0{\cdot}007789$. The increase of conductivity for the first 28 % haemolysis is 0·00023, and that for the remaining 72 % 0·000593. This is to be taken as an indication that the action of saponin is to liberate electrolyte from the cells unhaemolysed by the water, rather than to liberate retained electrolyte from the stroma of laked cells. It will be noted that the ratios 28 : 72 and 0·00023 : 0·00059 are very nearly the same, as they should be if our explanation is correct.

3. *Saponin haemolysis.*

We are able to confirm Stewart's finding that saponin liberates all the available electrolyte from the cells, provided, of course, that there is enough lysin present to produce complete haemolysis. If there is insufficient, an amount of electrolyte proportional to the number of cells haemolysed is liberated.

4. *Complement-amboceptor haemolysis.*

In this form of lysis, Stewart finds that the electrolytes are retained in the cells after the haemoglobin has escaped. Our results are shown in the following experiment:

	κ	
Normal saline	0·014240	
Suspension plus complement and amboceptor	0·01203	
increasing to	0·01211	
	0·01217	
and	0·01220	Complete lysis
Plus saponin	0·01307	

This is not dissimilar to the findings of Stewart. The lysis of cells by the complement-amboceptor system results in a very small change of conductivity—an increase of 0·00017—whilst the addition of saponin to the completely laked cells gives a further increase of 0·00087. On Stewart's hypothesis, this fact would be explained by a liberation of electrolyte from the ghosts by the saponin, it being considered that the passage of haemoglobin from the cells is not accompanied by the passage of any large portion of the electrolyte.

It is, however, possible that the saponin has some effect on the liberated haemoglobin. Stewart was unable to find evidence for the existence of a compound, split by dilution, between haemoglobin and electrolyte, but there may be such a compound nevertheless, and it may be split by saponin. It has been shown that the addition of saponin to saline produces no appreciable change of conductivity; it now remains to be shown whether or not the addition of saponin to haemoglobin, as derived from laked cells, produces any alteration.

The cells of the suspension used were laked by the addition of an appropriate amount of complement and amboceptor, and the laked fluid passed through a Berkefeld filter. In the filtrate, it may be taken that no ghosts were present; apart from dilution, on the other hand, the condition of the haemoglobin and the electrolyte would be unaltered. To this filtrate saponin was added, with the following result:

	κ
Filtrate	0·004864
Plus saponin	0·004961

This is an increase of about 2 %. It is to be noted that the concentration of this filtrate, both in electrolyte and in haemoglobin, is considerably less than that of the laked fluid resulting from the complement-amboceptor haemolysis, the concentration in the case of the filtrate being about one-third of that of the latter fluid; the 2 % alteration in conductivity on the addition of saponin is thus a very considerable one. Assuming, as appears necessary, that there is a compound between the haemoglobin and electrolyte, which is partly or completely broken down by the action of saponin, the change of conductivity in the fluid derived from complement-amboceptor haemolysis on the addition of saponin can be explained quite well without the necessity of postulating the liberation of electrolyte from haemolysed ghosts, for when we take into account the dilution of the filtrate, there is no difficulty in arriving at the 8 % alteration in the complement-amboceptor experiment.

This dilution, moreover, may explain why the alteration in the filtrate is as small as it is (2 %), for the dilution of the haemolysed fluid by water appears to be responsible for the splitting off of a certain amount of the electrolyte from the haemoglobin. In (2) above, the addition of saponin to cells partly laked by water does not appear to do more than lake the remaining cells—there is no necessity to call in an action on the haemoglobin to account for the final alteration in conductivity, presumably because the very addition of water has accomplished the greater part of the splitting. We do not wish, however, to lay stress on this point, for the differences are small; on the other hand, they are large enough to warrant attention being called to them, and to justify the suggestion that the true explanation of the results lies in there being a compound between haemoglobin and electrolyte, which compound is broken down, with the liberation of electrolyte, either by dilution with water, or by the addition of saponin.

5. *Heat haemolysis.*

We have been unable to confirm Stewart's results for the change of conductivity of heated suspensions, since we have found that heating suspensions or defibrinated blood to the temperature he mentions results in the formation of a thick fluid full of precipitate, and quite impossible to subject to accurate measurement. Even heating to much lower temperatures gives solutions which are very unsatisfactory for conductivity work. In this connection, however, one point may be recorded. The heating of a filtrate of laked cells is sufficient to change its conductivity appreciably, as the following figures show:

	κ
Filtrate	0·004864
Heated to 60°	0·004941

This is nearly a 2 % difference, and as it occurs with a dilute solution of haemoglobin, the changes occurring in defibrinated blood or in a concentrated cell suspension are such as cannot be ignored.

In this paper we have purposely omitted to make freezing-point measurements, as these are generally admitted to be very unreliable in experiments of this sort, and as such measurements, owing to their inferior accuracy, are of little value in checking conductivity estimations. We have also purposely ignored the points made by Brooks [1925] in his theoretical paper on the subject; not that we do not agree entirely with his conclusions, but because they do not affect the essential points which we wish to emphasise.

In order to enable anyone to convert the specific conductivities in which these results are expressed into actual resistances, we give the cell constant as 0·6976.

CONCLUSIONS.

1. The liberation of electrolyte from red cells during haemolysis by water is such as may be explained by each cell giving up the electrolyte contained in it, liberation of electrolyte being proportional to the percentage number of cells haemolysed, and complete when haemolysis is complete.

2. In the case of complement-amboceptor haemolysis, the full amount of electrolyte does not appear to be liberated. This is due to some of the electrolyte passing out of the cells combined with haemoglobin, from which it can be split off by the addition of saponin, and also, to some extent, by heating.

3. There appears no necessity to postulate that electrolyte is retained in any quantity in the haemolysed cells, after the liberation of haemoglobin.

This research was carried out during the tenure of a Crighton Research Scholarship by one of us (E. P.). The expenses were defrayed by a grant from the Royal Society.

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LXXXIII. THE INFLUENCE OF SMALL QUANTITIES OF POTASSIUM IODIDE ON THE ASSIMILATION OF NITROGEN, PHOSPHORUS AND CALCIUM IN THE GROWING PIG.

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The thyroid plays a significant part in the mechanism of growth and development, its secretion being "the most powerful stimulant of body metabolism known" [Sharpey-Schafer, 1924].

Baumann [1895] discovered that iodine is normally present in the thyroid gland and Kendall obtained from the gland a substance, thyroxin, in pure crystalline form which contains over 60 % of iodine and has been shown to possess most of the properties of thyroid extract [Kendall, 1919, 1, 2].

The notable work of Marine [1923] and his colleagues on the beneficial use of iodine in the treatment of endemic goitre in the human subject is well known. Clinical investigators report pathological changes in the thyroid glands of children who had died in a "marasmic" condition and note the beneficial effects accruing from thyroid extract treatment [Simpson, 1910].

Examples of thyroid diseases occurring in domestic animals are given by Ennis Smith [1917] who describes the condition known as "hairless pig malady," and by Kalkus [1920] who gives an account of goitre and associated conditions occurring in horses, cattle, pigs and sheep in certain districts of America. Both these workers emphasise the value of potassium iodide as a therapeutic agent in the conditions they describe.

Numerous workers have dealt with the action of thyroid extract and of thyroxin but there is little experimental work which measures in a quantitative manner the effect of simple iodine compounds on metabolism. A summary of the experimental work on the action of iodine or iodides is given by Barbour [1924], who refers to work on the influence of iodides on basal metabolism, the results of which were negative, and to work by Christoni who states that iodides may increase the excretion of urea, total nitrogen, uric acid, purine bases and chlorides.

In recent years Orr and his co-workers have been carrying out an extensive study of the mineral requirements of animals. Special attention has been paid by Husband, Godden and Richards [1923] to the influence of certain oils on the absorption and retention of nitrogen, calcium and phosphorus in growing pigs. Their results seem to indicate that cod-liver oil (which contains iodine) exercises a beneficial effect on the nitrogen as well as on the retention of calcium and phosphorus. Further unpublished data afford evidence that this beneficial effect on the nitrogen retention may be due, to some extent at least, to the iodine content of the cod-liver oil.

From the foregoing considerations it has been assumed that iodine is an essential constituent of every well-balanced diet, and it was decided to add small quantities of potassium iodide to normal cereal rations and observe the effect on the mineral metabolism of the growing animal with special reference to nitrogen, phosphorus and calcium.

EXPERIMENTAL.

Young male pigs from 2–4 months old were used. In each separate experiment the experimental and control pig were from the same litter and were comparable as regards weight and general condition. The methods, described by Husband, Godden and Richards [1923], for feeding the animals and for collection of excreta were followed throughout each experiment.

The urine and faeces were collected over 48-hour periods and duplicate analyses of nitrogen, phosphorus and calcium were made on each of those 2-day samples.

Four experiments were carried out. It was the aim to divide each into a "pre-period," feeding basal ration only, an "iodine-period" with basal ration plus potassium iodide, and a "post-period" during which the potassium iodide was withdrawn.

A control pig, on the basal ration only, was carried on simultaneously with each experimental pig, except in the case of Exp. 4. The average length of each period was 14 days.

Total nitrogen was determined by the Kjeldahl method; calcium by precipitation as oxalate and titration with $N/10$ $KMnO_4$; phosphorus by the Pemberton-Neumann method as described by Richards and Godden [1924].

Basal Ration.

The basal ration used for all pigs consisted of oatmeal, maize, blood-meal and either barley-meal (Exps. 1 and 2) or sharps (Exps. 3 and 4). The following figures show the average composition of the foodstuffs and the proportions in which they were used.

		Average composition %		
	Proportions	Total N	P_2O_5	CaO
Oatmeal	10 parts	2·08	1·09	0·080
Maize	10 ,,	1·45	0·69	0·009
Barley-meal or	10 ,,	1·29	0·82	0·062
Sharps	10 ,,	2·50	1·72	0·077
Blood-meal	1 part	13·58	0·35	0·147

The above ration is deficient in calcium and it has been the experience here that growing pigs break down if fed on this ration alone. Therefore, in order to supply calcium adequate to the need of a growing pig, powdered chalk or a solution of calcium chloride, or both were added to the ration in the amounts shown along with the data for each separate experiment. Olive oil was added to assist regular defaecation, and, where calcium chloride was used, 10 g. sugar were given to mask the bitter taste. Distilled water was given to drink *ad lib.*

In every case the iodine was added as a solution of potassium iodide. The exact intake of nitrogen, phosphorus and calcium is given along with the tables of experimental data (Tables I and II).

Exp. 1. The amount of iodine added daily to the ration of the experimental pig was 0·19 g. It was given in the form of 25 cc. of a 1 % solution of KI.

	Weight in kg.	
Number of pig	At beginning of experiment	At end of experiment
798 experimental	16·8	25·9
801 control	15·7	25·3

Daily ration fed to each pig.

Basal ration	775 g.
$CaCl_2$	25 cc. of 20 % solution ≡ 1·62 g. CaO
$CaCO_3$	6 g. ≡ 3·26 g. CaO

10 cc. olive oil and 10 g. sugar.

During the post-period the experimental animal had a tendency to eat its faeces, thus making the figures for that period somewhat unreliable.

Exp. 2. This experiment was similar to the first with the exception that the olive oil and calcium chloride were withdrawn. The amount of iodine added daily to the ration of the experimental pig was the same as in Exp. 1.

	Weight in kg.	
Number of pig	At beginning of experiment	At end of experiment
867 experimental	32·4	50·0
866 control	34·1	50·4

Daily ration fed to each pig.

Basal ration	1550 g.
$CaCO_3$	10 g. ≡ 5·68 g. CaO

As is shown in Tables I and II, it was clear from the first two experiments that the addition of iodine had some effect on the nitrogen and phosphorus metabolism. The generally positive nature of the results obtained in these two experiments made it desirable to test what effect a smaller dose of potassium iodide would have and to ascertain if possible what might be the minimum dose with which an effect could be obtained.

Exp. 3. In this experiment the amount of iodine added daily to the ration of the experimental pig was very much reduced. The amount was 25 cc. of 0·26 % KI solution ≡ 0·005 g. iodine.

	Weight in kg.	
Number of pig	At beginning of experiment	At end of experiment
1358 experimental	36·8	58·9
1359 control	32·8	54·3

Ration fed daily to each pig.

Basal ration	1550 g.
$CaCl_2$	50 cc. solution ≡ 3·248 g. CaO

10 cc. olive oil and 10 g. sugar

Exp. 4. This was a continuation of Exp. 3 and consisted in carrying on the control pig as an "iodine" pig. After this pig had acted as a control as far as the end of the iodine-period in Exp. 3, 0·003 g. iodine (as KI) was added daily for 12 days. No increase in the balances was observed so it was decided to double that amount (0·006 g. iodine). This still failed to produce any effect but maintained the balances at the same level. Again at the end of 10 days 0·5 g. KI (0·38 g. iodine) was added daily and this higher dose caused an immediate rise in all balances. There was no control in this case.

The experimental data relating to these four experiments are summarised in Tables I and II. Table I gives the data for the "iodine" pigs and Table II those for the corresponding controls.

Discussion of Results.

It seems advisable in discussing these results to group the four experiments together and lay emphasis on the aggregate effect over four experiments, rather than to take each one individually. In the following table the average retention of nitrogen, phosphorus and calcium in the pre-period has been subtracted from that in the iodine-period in each of the four experiments, a plus sign indicating an increased retention in the iodine-period and a minus sign indicating a fall in that period. For the control pigs a similar procedure has been adopted, the averages for a period corresponding to the pre-period in the experimental pigs being subtracted from the period corresponding to the iodine-period. The average increase or decrease in retention per pig in the iodine-period is shown at the foot of each column. In Exp. 4, the retention during the 0·006 g. iodine-period is subtracted from that during the 0·38 g. iodine-period.

From a study of Tables I, II and III it is apparent that on adding iodine an increase in the retention of nitrogen and phosphorus is the rule rather than the exception, whereas in the control pigs without iodine a fall in these balances is more general. The effect on the calcium balance is more difficult to gauge.

EXPERIMENTAL PIGS

Period	Days	Average daily weight faeces	Nitrogen (N)					Phosphorus (P_2O_5)					Calcium (CaO)				
			Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
Pig No. 798. Exp. 1																	
Pre-period	12	510	15·80	5·41	4·80	10·21	5·59	6·74	0·13	3·80	3·93	2·81	5·27	0·48	2·77	3·25	2·02
KI-period	12	425	15·80	5·57	4·17	9·74	6·06	6·74	0·12	4·02	4·14	2·60	5·27	0·87	2·06	2·93	2·34
Post-period	10	285	15·80	5·50	3·30	8·80	7·00	6·74	0·22	2·72	2·94	3·80	5·27	0·87	1·45	2·32	2·95
Pig No. 867. Exp. 2																	
Pre-period	14	631	31·77	11·30	8·91	20·21	11·56	13·85	1·51	7·50	9·01	4·84	6·50	0·16	3·38	3·54	2·96
KI-period	14	587	31·77	11·34	8·12	19·46	12·31	13·85	1·81	6·61	8·42	5·43	6·50	0·16	2·71	2·87	3·63
Post-period	14	585	31·77	12·24	8·08	20·32	11·45	13·85	2·02	6·84	8·86	4·99	6·50	0·19	2·97	3·16	3·34
Pig No. 1358. Exp. 3																	
Pre-period	16	560	35·87	14·45	8·61	23·06	12·81	17·83	5·04	10·42	15·46	2·37	4·18	0·10	3·04	3·14	1·04
KI-period	16	495	35·87	14·80	7·22	22·02	13·85	17·83	5·24	9·76	15·00	2·83	4·18	0·09	2·28	2·37	1·81
Post-period	12	463	35·87	16·40	6·82	23·22	12·65	17·83	5·51	9·47	14·98	2·85	4·18	0·10	2·00	2·10	2·08
Pig No. 1359. Exp. 4. (Control pig from Exp. 3 continued as an experimental pig.)																	
0·003 I_2-period	12	600	35·87	15·51	7·47	22·98	12·89	17·83	5·17	10·73	15·90	1·93	4·18	0·11	2·09	2·20	1·98
0·006 I_2-period	10	636	36·73	16·59	7·51	24·10	12·63	18·53	4·83	11·64	16·47	2·06	4·31	0·10	2·44	2·54	1·77
0·5 g. KI-period	10	620	36·73	16·48	6·73	23·21	13·52	18·53	4·36	10·09	14·45	4·08	4·31	0·09	2·14	2·23	2·08

[1] In averaging, the first 2 days of the KI-period are included in the pre-period and the first 2 days of the post-period are included in the iodine-period.

Table II. *Average daily excretions and balances in g.*

CONTROL PIGS.

Period	Days	Average daily weight faeces	Nitrogen (N)					Phosphorus (P_2O_5)					Calcium (CaO)				
			Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance	Intake	Urine	Faeces	Total	Balance
Pig No. 801. Exp. 1																	
Pre-period corresponding to	12	391	15·80	5·71	4·51	10·22	5·58	6·74	0·09	3·81	3·90	2·84	5·27	0·66	2·34	3·00	2·27
KI-period	12	342	15·80	5·85	4·01	9·86	5·94	6·74	0·08	3·69	3·77	2·94	5·27	0·71	2·16	2·87	2·40
Post-period	10	353	15·80	6·02	3·90	9·92	5·88	6·74	0·09	4·07	4·16	2·58	5·27	0·69	1·84	2·53	2·74
Pig No. 866. Exp. 2																	
Pre-period corresponding to	14	600	31·77	11·07	8·58	19·65	12·12	13·85	1·58	7·40	8·98	4·87	6·50	0·23	3·46	3·69	2·81
KI-period	14	680	31·77	11·41	9·23	20·64	11·13	13·85	1·36	7·70	9·06	4·79	6·50	0·16	3·00	3·16	3·34
Post-period	14	650	31·77	11·72	9·03	20·75	11·02	13·85	1·43	7·67	9·10	4·75	6·50	0·14	2·50	2·64	3·86
Pig No. 1359. Exp. 3																	
Pre-period corresponding to	16	631	35·87	14·10	9·13	23·23	12·64	17·83	4·96	10·10	15·06	2·77	4·18	0·14	2·73	2·87	1·31
KI-period	16	622	35·87	15·21	8·05	23·26	12·61	17·83	5·18	10·62	15·80	2·03	4·18	0·12	2·19	2·31	1·87
Post-period[2]	12	600	35·87	15·51	7·47	22·98	12·89	17·83	5·17	10·73	15·90	1·93	4·18	0·11	2·09	2·20	1·98

[2] This is also the 0·003 g. iodine-period in Exp. 4.

Table III. *The average increase or decrease (in g.) in retention of nitrogen, phosphorus and calcium during the iodine-period.*

	Experimental pigs.			Control pigs.		
Exp.	Nitrogen	P_2O_5	CaO	Nitrogen	P_2O_5	CaO
1	+0·47	−0·21	+0·32	+0·36	+0·10	+0·13
2	+0·75	+0·59	+0·67	−0·99	−0·08	+0·53
3	+1·04	+0·46	+0·77	−0·03	−0·74	+0·56
4	+0·89	+2·02	+0·31		No control	
Total	+3·15	+2·86	+2·07	−0·66	−0·72	+1·22
Average	+0·78	+0·71	+0·52	−0·22	−0·24	+0·41

Nitrogen Balance.

That the action of iodine on the nitrogen metabolism has an immediate rather than a gradual effect will be seen from a consideration of Table IV.

Here the figures for the nitrogen balances are given in greater detail. The retention over the last 8 days of the pre-period is contrasted with the retention over the first 8 days of the iodine-period, figures for separate 2-day analysis periods being shown. It is clear that the increase in retention takes place almost immediately on the introduction of iodine. In Exp. 4 the last 8 days of the 0·006 g. iodine-period are compared with the first 8 days of the 0·38 g. iodine-period.

Table IV. *Average daily retention of nitrogen in g.*

	Exp. no.			
	1	2	3	4
Pre-period, last 8 days	5·50	10·75	14·37	13·29
	5·26	11·77	13·37	13·24
	5·64	13·09	12·49	11·63
	5·47	10·46	10·94	12·39
——→				
Iodine-period, first 8 days	6·00	12·30	12·56	13·73
	6·16	13·04	14·57	13·47
	5·28	11·36	14·12	13·52
	6·60	12·21	13·94	12·81

NOTE. ——→ indicates introduction of iodine.

Exps. 2 and 3 are the only ones in which a consideration of the post-period is legitimate. The post-period in Exp. 1 is not considered since during this period the animal had a tendency to eat its faeces thus rendering the results unreliable; and in Exp. 4 no post-period without iodine was studied.

It will be seen from Table I that, on the removal of iodine in Exps. 2 and 3, the average retention of nitrogen in the post-period fell back almost to the same figure as the average retention in the pre-period.

Undoubtedly one of the functions of the thyroid gland is intimately connected with the capacity of the animal for proper growth, though the mechanism of this process is as yet unknown. The increased retention of nitrogen after the addition of iodine, observed in all these experiments, is an index of the inducement of better growth probably due to the stimulation of the thyroid gland by the iodine. Schafer [1912] has shown that addition of small amounts of thyroid tissue to the normal dietary of growing rats causes an acceleration of growth and retention of nitrogen in the body. Though this is an experiment not strictly comparable with the feeding of iodine *per se*, the similar results obtained for the nitrogen balances in the experiments described show that no doubt the action of the thyroid tissue is in part due to its iodine content. Hesse [1924] from some 30 metabolism experiments on dogs found that on feeding sodium iodide increased excretion of nitrogen in the urine could always be produced. From Table I it will be seen that our results, in the main, confirm this finding. Hesse concludes from this that body protein is broken down, and that analyses of urine showing increased elimination of nitrogen are sufficient evidence for stating that there is a general decrease in the retention of nitrogen in the body. This may be so in full-grown animals, but it is unfortunate that no analyses of faeces are given by Hesse for without them erroneous conclusions are apt to be drawn.

Many other workers refer to the growth-promoting effects of feeding alkali iodides or iodine in organic form, though these observations, made without actual measurement, are less precise.

Hunziker [1920] noted a marked difference in the height of children given iodine from that of children not exposed to its influence. Horses suffering from lymphangitis when fed on sea-weed, which is rich in iodine, put on weight remarkably and were restored to normal health [Adrian, 1918].

In a feeding experiment on calves carried out on the farm at this Institute it has been found that a group of 12 calves getting potassium iodide in their ration gained, in the same time, three and a half hundredweights more than a similar group which had no iodine.

In our experiments the periods are too short to admit of a comparison between rates of growth or increase in weight in one period over another, but it is significant that throughout the whole experiment the "iodine" pigs grew generally better than the controls with the exception of those in Exp. 1.

Table V shows the total increase in weight during the entire experiment. It will be seen that in two cases the "iodine" pigs put on an average of 1 kg. more than the controls in the same time.

Table V. *Total increase in weight (kg.) during each entire experiment.*

	Exp. no.		
	1	2	3
Experimental pigs	9·1	17·6	22·1
Control pigs	9·6	16·3	21·5

Phosphorus Balance.

Corresponding with the nitrogen figures there is an increase in the retention of phosphorus on the addition of iodine to the ration. This is not so marked as in the case of nitrogen, but that it is none the less definite is seen when one compares the phosphorus balances in the "iodine" pigs with those of the controls. Leaving out Exp. 4, where the rise in the phosphorus balance seems abnormally high, we find that in experimental pigs there is an average increase in retention of 0·28 g. in the iodine-period over that in the pre-period, whereas, in the controls, with the exception of Exp. 1, for a corresponding period a decrease in retention took place. The average retention of phosphorus in the post-period, in Exp. 2, fell back to a figure comparable with that in the pre-period, whilst in Exp. 3 no such drop was apparent, the retention being maintained as in the iodine-period.

Calcium Balance.

In every case the calcium balance throughout the entire experiment was on the ascendant in both "iodine" and control animals, thus making any further increment, which might possibly be due to iodine, difficult to observe. On the average, however, as seen in Table III, the increased retention during the iodine-period in the experimental pigs was higher than the increase for a corresponding period in the controls.

Amount of Iodine required to give a Positive Result.

The daily amount of potassium iodide used in these experiments varied from 0·5–0·0039 g. (from 0·38–0·003 g. iodine). In Exps. 1 and 2 the amounts of actual iodine used were equivalent to 0·025 % of the ration, and in Exp. 3 to 0·00032 %. In Exp. 4 a dose equal to 0·00019 % of the food failed to produce an effect. This was doubled and fed for 10 days, but it was not until the percentage was raised to 0·025 g., as in Exps. 1 and 2, that an effect became apparent. Sufficient experiments using varying amounts have not been carried out to enable us to state definitely what is the least amount with which one can obtain an effect. The animals used in these experiments had not been fed with any iodine-rich food (such as fish-meal) before they were used for the present study and in considering the results it must be borne in mind that in some cases the iodine store in the thyroid gland might not be so high as in others.

Uncompleted studies on the iodine content of thyroid glands of pigs show marked variation in the amounts stored; and in the present experiments it is legitimate to assume that individual animals would react differently to different doses, those with a depleted initial store requiring only a minute dose to give an effect, whilst exceedingly healthy animals, such as the pig used in Exp. 4, seem to require a relatively large dose before any change becomes apparent. No toxic symptoms due to iodine were observable in any of the animals used.

These results point to the necessity for ensuring that a sufficiency of iodine is present in synthetic rations which are presumed to contain all the known essential constituents of food. This precaution, which apparently has not been taken in some experimental work published in recent years, is of special importance when cod-liver oil and green food are being used. The physiological effects of the unknown constituents of cod-liver oil are being widely investigated at the present time; and the experiments described in this paper seemed desirable when one considers that this oil is relatively very rich in iodine, containing on an average 0·001 %. Weight for weight this is 1000 times more than is usually present in the commoner foodstuffs such as cereals where, as a rule, it is present only in traces [Forbes, 1916]. Green vegetables occupy an intermediate position between cod-liver oil and cereals as regards their iodine content, and Forbes concludes that they are the most important source of iodine in the human dietary. They contain about one-hundredth of the amount usually present in cod-liver oil, though Fellenberg [1923] estimates the iodine content of some green vegetables at more than a thousandth of that amount.

McCarrison [1920], in certain of his experiments on deficiency diseases, describes the effect of onions in restraining the tendency to thyroid hyperplasia in pigeons which were fed on a deficient diet. He states that this effect may have been due to the enrichment of the dietary in iodine which onions may have afforded, though he is not inclined to support that view. It is significant, however, that onions stand fairly high among vegetables in their iodine content [Forbes, 1916]. It is, of course, recognised that the well-known effects on nutrition of cod-liver oil and green food cannot be attributed entirely to iodine; but it is suggested that when these substances are added to iodine-free rations the possible effect of their iodine content should be considered.

SUMMARY.

1. Small amounts of iodine, as potassium iodide, added to the cereal rations used, led to an increased assimilation and retention of nitrogen and phosphorus in young growing pigs. Though the nitrogen and phosphorus retentions ran parallel to each other the effect of iodine was usually more marked in the case of nitrogen.

2. There was also some evidence of an increased retention of calcium, but this was less definite.

3. The amount of iodine required to obtain this result varies with individual animals, and probably depends on the type of diet fed previous to the experiment.

I desire to express my indebtedness to Dr J. B. Orr, Director of the Rowett Institute, who suggested this work; also to Mr Godden in whose department the work was carried out.

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LXXXIV. THE EFFECT OF ULTRA-VIOLET LIGHT ON THE MINERAL METABOLISM OF THE LACTATING ANIMAL.

(PRELIMINARY COMMUNICATION.)

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During the past few years a considerable amount of work has been done on the mineral metabolism of lactating animals. A review of the recent literature is given by Meigs [1922].

It has been found that, during a large part of the period of lactation, the heavy milking cow usually suffers a loss of certain mineral elements from her body. The negative balance of calcium may continue even though there is an abundance of this element in the ration [Forbes and others, 1922]. There is evidence to show that privation of calcium adversely affects reproduction in cows [Hart, Steenbock and Humphrey, 1920], and it has been suggested that the disturbance of equilibrium in mineral metabolism is a predisposing cause, not only of sterility and abortion, but also of several other diseases to which dairy cows seem specially liable.

During the last few years work on metabolism in lactation has been carried out at this Institute. Some of the data which have been accumulated seem to indicate that the rate of assimilation of calcium and phosphorus is greater in summer than in winter, and the difference cannot be wholly accounted for by variations in the composition of the diet. Recent work on the influence of ultra-violet rays on nutrition suggested that these rays might be the unknown factor, and that they might have an influence on the assimilation of calcium and phosphorus in the lactating animal. The results of some preliminary work showed that the exposure of cows to ultra-violet rays from a carbon arc lamp had no marked immediate effect on milk yield. In work with growing pigs, however, it was found that, on certain diets at least, exposure to the rays of the carbon arc lamp very markedly increased calcium and phosphorus retention [Orr, Henderson and Magee, 1924; Henderson, 1925]. It was thought that ultra-violet rays might have a similar influence in mature animals with a very active mineral metabolism. An investigation on the effects of ultra-violet rays on the calcium, phosphorus and nitrogen balances during lactation was therefore carried out.

It was originally intended to defer publication of the results referred to in this paper until certain other investigations of a kindred nature were completed.

Meanwhile, however, Hart, Steenbock and Elvehjem [1924] have published results which show that ultra-violet rays may convert negative balances of calcium and phosphorus in the lactating goat to positive balances. As this influence of ultra-violet rays on the mineral metabolism during lactation is of very great potential practical importance, it was thought that the present note confirming the findings of these workers should be published.

EXPERIMENTAL.

This note deals only with the calcium balance, which is the one of greatest practical importance, and gives the details of only one experiment. Figures summarising the results of the other two experiments which differ only in detail are included in Table III.

Methods. The experimental work was done on goats as they are more convenient subjects for balance experiments than cows.

The diet consisted of 900 g. hay, 300 g. oatmeal and 50 g. linseed meal. Preliminary tests showed that this diet, both in composition and amount, suited the goat's appetite at the beginning of the experimental period. During the period of irradiation the appetite improved and 100 g. oatmeal per day were added to the diet from the sixth day of irradiation onwards. The necessary precautions were taken to ensure that the exact intake of calcium and phosphorus for each day should be known.

The goat was kept in a pen for 3 weeks before the experiment was begun, to accustom it to confinement and to the experimental ration. It was then confined to a metabolism cage. Collection and analysis of excreta were carried out for a total period of 54 days. From the 13th to the 34th day the animal was irradiated for a total period of 4 hours per day at a distance of 4 feet, about a sixth of the animal's body being exposed to the rays at a time.

RESULTS.

Table I. *Calcium balance in g. for three-day periods.*

Periods	Intake Ca	Output Ca	Balance Ca
Pre-period	15·09	16·33	−1·24
	15·09	16·60	−1·51
	14·75	14·60	+0·15
	14·92	15·09	−0·17
Irradiation period	15·60	15·35	+0·25
	15·58	12·59	+2·99
	15·89	15·75	+0·14
	15·85	16·24	−0·39
	15·89	17·05	−1·16
	15·89	14·72	+1·17
	15·89	14·84	+1·05
Post-period	15·78	16·02	−0·24
	15·72	15·85	−0·13
	15·89	18·24	−2·35
	15·89	17·46	−1·57
	15·89	16·28	−0·39
	15·89	16·29	−0·40
	15·89	15·43	+0·46

Table II. *Average daily balances (Ca in g.) in the pre-, irradiation- and post-periods.*

Pre-period	Irradiation period	Post-period
−0·24	+0·16	−0·22

Table III. *Alteration in the balances produced by irradiation.*

Daily averages in g. Ca		Exp. 1		Exp. 2		Exp. 3	
		Pre-period	Irradiation period	Pre-period	Irradiation period	Pre-period	Irradiation period
Intake		4·98	5·27	8·38	8·38	6·55	6·53
Output	Faeces	3·97	3·85	8·41	8·24	6·20	5·80
	Urine	0·21	0·20	0·09	0·09	0·29	0·30
	Milk	1·04	1·07	0·29	0·25	0·30	0·44
	Total	5·22	5·11	8·79	8·58	6·80	6·54
Balance		−0·24	+0·16	−0·41	−0·20	−0·25	−0·01

Table IV. *G. of calcium per* 100 *cc. milk (first and third experiments*).*

Pre-period	Irradiation period	Post-period
0·168	0·171	0·167
0·177	0·184	0·181

* The figures for Exp. 2 are not available, as washings with distilled water were added to the milk before analysis.

DISCUSSION.

In the first experiment, under the influence of the irradiation an average negative balance of 0·24 g. per day was changed to a positive balance of 0·16. In the second a negative balance of 0·41 was reduced to 0·20, and in the third a negative balance of 0·26 was reduced to 0·01. In Exps. 1 and 3 these reduced net losses of calcium occurred concurrently with increased secretion of milk. The gain in calcium absorption and retention is almost entirely accounted for by reduced excretion in the faeces, as is shown in the following table.

Table V. *Ca intake minus excretion in faeces (average per day in g.).*

Period	Exp. 1	Exp. 2	Exp. 3
Pre-period	1·01	−0·03	0·35
Irradiation period	1·42	0·14	0·73

Thus, under the influence of irradiation, there is either increased absorption from the intestine or decreased excretion into the intestine. The same result was obtained by us in growing pigs subjected to irradiation [Orr, Henderson and Magee, 1924]. This decreased excretion of calcium in the faeces under the influence of irradiation has since been noted by Hart, Steenbock and Elvehjem [1924] in goats, and by Webster and Hill [1925] in rats.

It has been generally believed that calcium is excreted chiefly by the large intestine. The work of Telfer [1923], however, makes it appear doubtful whether, normally, calcium is excreted into the intestine in appreciable amounts. In his experiments he regards the calcium of the faeces as chiefly, if not entirely, unabsorbed residue. The work of our colleagues and ourselves on the factors affecting the retention of calcium and phosphorus in growing animals tends to confirm Telfer's conclusion. The results of the above experiments, therefore, afford further evidence in favour of the view previously put forward, that the beneficial effect of ultra-violet light and also of oils on the assimilation of calcium is probably due chiefly to their influence in increasing the percentage of calcium which is absorbed from the intestine [Husband, Godden and Richards, 1923; Orr, 1924; Henderson, 1925].

There was a slight increase in the percentage of calcium in the milk during the period of irradiation. The data are too meagre to warrant any definite conclusion. They are interesting, however, in the light of results obtained by Hess, Unger and Supplee [1920]. These workers found a marked difference between the percentage composition of the ash of milk of stall-fed cows on an antiscorbutic-free diet and that of the same cows on pasture. The percentage of calcium in the former case was 0·138 (as CaO) and in the latter 0·165. These workers, while not expressing any definite opinion, suggested that the difference might possibly be due to the antiscorbutic vitamin of the pasture. In view of our results it is probable that the difference noted by them may have been due, partly at least, to the influence of sunlight while the cows were on pasture.

SUMMARY.

In three balance experiments on lactating goats it was found that irradiation of the animals by the carbon arc lamp reduced the loss of calcium from the body.

Leaving out of consideration the amounts contained in the milk secreted, the reduction was entirely accounted for by decreased excretion in the faeces; the urinary excretion remaining practically constant.

It is suggested, therefore, that irradiation by the carbon arc lamp caused an increased absorption of calcium from the intestine.

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LXXXV. A MICRO-METHOD FOR TITRATING THE BICARBONATE IN PLASMA.

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(*Received June 2nd, 1925.*)

In the early method of Zuntz [1867] and its various modifications by Lassar [1874], Jaquet [1892], Loewy [1894], Wright [1897] and Snapper [1913] a dilute solution of acid was added to a known volume of serum until a drop of the mixture changed the tint of an indicator paper, litmus, lakmoid or neutral red as the case might be. The particular end-point depended upon the predilection of the observer. In terms of modern physical chemistry, the serum was titrated to an arbitrarily chosen p_H.

Hans Meyer [1883] pointed out 42 years ago that the alkalinity of serum, determined in this way, was a function of the indicator employed, and further that the amphoteric proteins took part in the reaction. He came to the conclusion that the only definite and important measurement of blood alkalinity was its CO_2 combining power. The general use of the gasometric method of Van Slyke and Cullen [1917] indicates the recognition of the soundness of Meyer's reasoning.

It is difficult to say what is the p_H of a drop of serum which just changes the colour of red litmus paper because of the considerable "salt" error of azolitmin. Kolthoff [1922] found the error to be as much as $+ 1{\cdot}0\ p_H$ in the presence of 0·5 normal NaCl; that is the hydrogen ion concentration was ten times what the colour indicated. The more the NaCl is concentrated in the drop by evaporation the greater will be the salt error. We find that a sensitive glazed red litmus paper is slightly blued when a drop of $M/15$ phosphate solution of p_H 6·50 remains upon it for a few minutes, but is not allowed to dry. A similar solution of p_H 6·45 does not produce an appreciable alteration of colour. It would appear, therefore, that one is titrating to an end-point of p_H 6·47 but as the phosphates are concentrating all the time in the drop, the real p_H will be a little lower from the increasing "salt error" of the azolitmin.

Titration even to 6·5 indicates an alkalinity of the blood considerably greater than that determined gasometrically. In addition to the base balanced by HCO_3' a variable amount associated with the proteins is titrated, depending upon the litmus paper used, the time allowed for the CO_2 to escape from the drop and the degree of change in tint which the observer decides upon as his end-point.

Although these indicator-paper methods afford useful comparative information, a method which only measures the base present as bicarbonate is preferable. Granted that the proteins, especially haemoglobin, are mostly responsible for the buffer action, it is only the alkaline bicarbonate and the respiration which are available for *adjusting* the reaction of the blood. Consequently, during recent years, the gasometric method of Van Slyke and Cullen [1917] has been generally adopted.

The method about to be described was devised by one of us, whilst abroad during the war, to measure the bicarbonate in blood-serum under circumstances in which the means to carry out Van Slyke and Cullen's method were not available.

It seemed likely that by titrating a small quantity of serum diluted with NaCl solution, and shaking out the CO_2 meanwhile, an end-point could be found at which the value obtained corresponded with the amount of alkali, as bicarbonate, determined gasometrically after the serum had been brought into equilibrium with alveolar air. After trial of titration to different end-points, it was found that when the indicator used was neutral red, the end-point sought was when the colour matched that of a $M/15$ solution of phosphates of p_H 7·07 to which a similar amount of indicator had been added. That the neutral point is the most suitable end-point is fortuitous. The reaction is a little acid of this, as neutral red has a small protein error. The real p_H is about 7·0.

The method was demonstrated at a meeting of the Biochemical Society in November, 1918, but was not published at the time because neutral red was not an entirely satisfactory indicator owing to gradual precipitation when the neutral point was approached. It was hoped to overcome this difficulty or to substitute another indicator. We have not been able to find another indicator to replace neutral red but have succeeded in obviating the separation

Table I.

The volumes per cent. of CO_2 in plasmas calculated from the titration values compared with those found by Van Slyke's method. 0·25 cc. was used for the titration and 1·0 c.c. for the gasometric estimation.

Specimen	CO_2 calculated	Van Slyke CO_2 observed
Smith plasma	60·0	61·2
Horse serum	41·0	43·3
1229 plasma	58·0	57·6
1372 ,,	59·5	58·5
1890 ,,	66·0	64·8
1890 B ,,	66·0	63·0
Spencer ,,†	70·0	65·0
Roper ,,	65·0	66·9
Hawes ,,	68·0	66·0
Simkins ,,	68·0	68·5
Bass ,,	59·0	61·9
Pearce ,,	66·0	63·9
Platt ,,	62·0	63·0

† This plasma was haemolysed.

of the indicator by titrating in the presence of 16 % alcohol. This amount of alcohol does not appreciably alter the sensitivity of neutral red to hydrogen ions and facilitates the shaking out of CO_2 by lessening frothing.

During the last few years we have used the method in clinical and experimental work and found it handy and reliable. Its merits are the simplicity of the apparatus and the small quantity of serum (0·25 cc.) required. It affords results which agree with the gasometric with an error not exceeding ± 5 % as is seen from the observations in Table I. In these the gasometric estimations were made by one observer and the titrations by another.

Meanwhile, Van Slyke, Stillman and Cullen [1919] have described a titration method, with neutral red as indicator, as an alternative to the gasometric method. Their method differs somewhat from ours. To 2 cc. of plasma, 5 cc., *i.e.* excess, of 0·02 N HCl is added and the CO_2 shaken out. The acidified serum is diluted with 20 cc. of water and titrated back with 0·02 N NaOH to an end-point corresponding with the colour of $M/8·5$ phosphate solution of p_H 7·4. The results recorded agree with the gasometric titration within 7 %.

Van Slyke [1922] has recently elaborated this method and increased its accuracy by using the actual plasma, collected without loss of CO_2 and diluted to a similar extent, as the standard to titrate to. This procedure obviates a number of small errors and renders the method unexceptionable, but the determination cannot be carried out upon less than 10 cc. of blood.

Experimental Procedure.

Collection of the samples of blood. The blood is drawn from the ear into a collecting tube made from a piece of glass tube of 7 mm. internal bore, sealed at one end and drawn out at the other until the internal bore is about 1 mm. The length from the sealed end to the shoulder should be about 4 cm. It will then conveniently contain up to 1 cc. of blood. The collecting tube must have a small hole blown near the drawn-out end to permit the air to escape as the blood enters (see Fig. 2). Before use a few crystals of oxalate and a tenth of a cc. of liquid paraffin are introduced into it. A free puncture of the skin is made and the capillary end applied to the drop of blood, which then flows into the tube and under the paraffin. The plasma is separated from the corpuscles by centrifuging.

The amount of CO_2 lost and consequent emigration of H and Cl ions out of the corpuscles by collecting blood in this way is not sufficient to introduce a serious error. The discrepancy would appear to be about 2 %. It was determined by the following experiments in which the amount of exposure to air closely simulated that which occurs when blood is drawn from the ear.

A piece of rubber pressure tubing bearing a screw clip was placed on the upper end of a 1 cc. pipette; the tip was put into some blood which had been collected under paraffin and the pipette filled by suction. The screw was tightened and the pipette clamped in a holder. By relaxing the screw clip the blood was allowed to escape from the pipette in drops. As each drop formed it was run into one of the blood-collecting tubes under paraffin. When sufficient blood had been collected in the tube the plasma was separated by centrifuging. At the same time some of the original blood was centrifuged. Observations were made upon four samples of blood. In Table II below, the titration values, expressed as volumes CO_2 % in the plasmas, are set forth.

Table II. *Volumes* CO_2 %.

Sample	Blood taken direct	Same blood allowed to drop from pipette
1	51·0	49·6
2	55·4	54·5
3	64·5	63·0
4 *a*	40·0	39·6
4 *b*	40·7	39·6

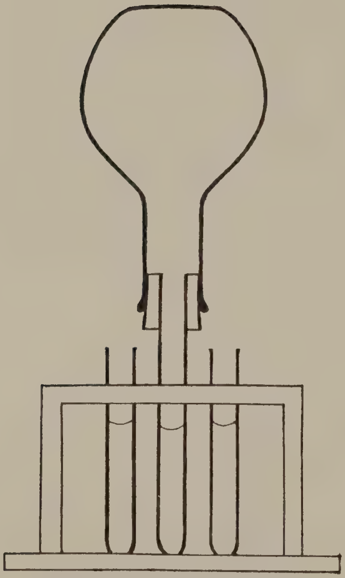

Fig. 1. Colorimeter stand.

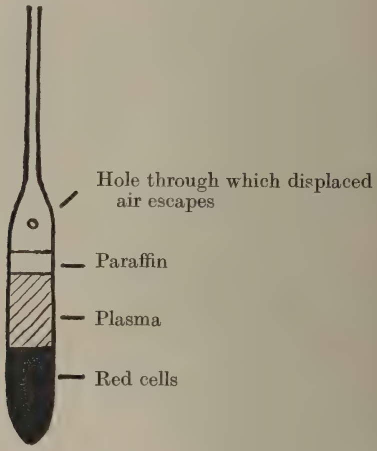

Fig. 2.

The amount of bicarbonate in blood drawn from the ear by a free puncture is lower than in venous blood. In this respect capillary blood approximates more closely to arterial blood, as Lündsgaard and Moller [1922] found for oxygen content.

Blood drawn in this way gives sufficiently consistent results, as the following observations show. The blood was collected from the finger at intervals of about 15 minutes and the plasma titrated.

Sample	Length of time taken in collection	Vols. % CO_2
1	1·0 cc. in 10 minutes	59
2	1·0 „ 5 „	62
3	0·8 „ 5 „	62
4	0·8 „ 5 „	61

However the blood be obtained, if the titration is not proceeded with at once, the plasma is best stored in tubes coated with paraffin wax.

Apparatus required for the titration.

1. A few test tubes of equal bore made from the same piece of clear white resistance-glass. Convenient dimensions are 8 cm. long and an internal diameter of 8 mm.

2. A 100 cc. flask of hard glass (*e.g.* Jena) with rubber stopper[1] bored to take one of the test tubes.

3. A micro-burette: a 1 cc. pipette graduated in 100ths with rubber tube, nozzle and pinch-cock answers the purpose.

4. A pipette graduated to contain 0·25 cc.

5. A simple colorimeter (Fig. 1) shaped like a short test tube rack with three holes to take the test tubes. Suitable dimensions are 9 cm. long, 6 cm. high and 5 cm. wide. The inner surface is blackened and one of the open sides is covered with a tissue paper screen. The holes are best placed as close as possible to the paper screen.

Chemicals required.

1. Standard $N/10$ H_2SO_4 from which $N/100$ can be made up as required.
2. Standard phosphate of p_H 7·07 made up as follows:

Potassium dihydrogen phosphate $M/5$	33·33 cc.
Caustic soda $N/10$	43·66 cc.
CO_2-free distilled water to	100 cc.

3. Neutral red 0·05 % dissolved in water.
4. Sodium chloride 3·5 %.
5. Alcohol 40 %.
6. A solution of burnt sugar which has been carefully neutralised and to which toluene has been added to prevent moulds growing in it.

Details of the titration.

0·25 cc. of plasma is drawn up into the graduated pipette and delivered into the flask. The pipette is washed out to the mark three times with the saline. 1 cc. of the 40 % alcohol is then added, so that the flask contains 2 cc. The contents are mixed and the rubber stopper carrying one of the test tubes inserted. The flask is inverted, so that the contents run down into the test tube, and placed in this position in the colorimeter.

2 cc. of the phosphate solution are placed in another of the tubes and this is put in the colorimeter beside the plasma. A small quantity of the solution of burnt sugar is then added to it with a fine dropping pipette until it matches the colour of the plasma. Another cc. of phosphate is added and five to eight drops of the indicator, the depth of colour chosen depending on personal preference.

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[1] The stoppers must be clean. They should be moistened with petrol and rubbed with a rag until all sulphur is removed. Subsequently they should be boiled first in soda, then in weak acid and thoroughly washed with distilled water.

Acid is then run into the flask from the micro-burette until the contents become bright pink. The fluid is well shaken by swirling it round the flask, the air being frequently changed by inserting a piece of wide bore glass tubing and inhaling through it. Exhalation in the immediate neighbourhood of the flask must be avoided. As the CO_2 is removed the colour of the fluid changes to an orange tint again. The shaking out process is repeated after each addition of acid. As the end-point approaches, single drops of acid are added and the stopper is placed in the flask, the flask inverted and the colour of the contents compared with that of the phosphate tube.

The amount of acid added when the plasma is just alkaline to the phosphate, matches it, and is just acid are noted, and the mean between the first and last readings is taken as the end-point. With a little practice the alkaline and acid points lie 0·01 on either side of the match. Much more shaking is required to get rid of the last traces of CO_2 than might be anticipated. It is necessary to continue to add acid until the fluid remains definitely pink and it is impossible to alter the colour by further shaking and aeration.

If the end-point is accidentally overshot it is possible to titrate back with $M/50$ sodium bicarbonate, shaking out meanwhile. Sodium hydroxide *must not* be used, as it is difficult to shake in CO_2 from atmospheric air in a reasonable length of time.

DISCUSSION.

Our method is frankly empirical but it is appropriate to discuss why it is necessary to titrate to an apparent end-point of p_H 7·07 (really p_H 7·0) to arrive at the alkali present as bicarbonate instead of to the p_H of blood which is about 7·3 after cooling to 18°.

The reason appears to be that the whole of the bicarbonate cannot be estimated unless the air with which it is brought into equilibrium is entirely free from CO_2. In the dilute solutions of bicarbonate with which we are working the small amount left undetermined is very significant. If, on the other hand, the solution is entirely freed from CO_2 and bicarbonate, by acidifying and shaking out the CO_2, practically the whole of the bicarbonate can be estimated by titrating back to the p_H of blood with NaOH. This is the principle of the method of Van Slyke, Stillman and Cullen [1919].

The amount of bicarbonate untitrated, when 0·25 cc. of $0·02M$ $NaHCO_3$ made up to 2·5 cc. with saline is estimated by our method can be derived from Hasselbalch's [1917] formula

$$p_H = p_{K_1} + \log \frac{[\text{Bic.}]}{[CO_2]}.$$

Let us assume that the air of the laboratory contains 0·05 % CO_2 and that the titration is carried to an end-point of p_H 7·4 and the final volume is 3 cc.

Taking the value of p_{K_1} at 18° as 6·22, which is what we have found for

$0{\cdot}02M$ $NaHCO_3$ in the presence of 0·85 % NaCl (Warburg [1922] found 6·24 under similar circumstances) we have

$$\log \frac{[\text{Bic.}]}{[CO_2]} = 1\ 2,$$

or

$$[\text{Bicarbonate}] = 13{\cdot}1 \times [CO_2].$$

The molecular concentration of CO_2 in water at 18° which has been brought into equilibrium with air containing 0·05 % CO_2 is

$$\frac{0{\cdot}05 \times a^{18^\circ}\ CO_2{}^*}{100 \times 22{\cdot}3} = 0{\cdot}000021.$$

Multiplying by 13·1 gives 0·000275 for the molecular concentration of the bicarbonate in the 3 cc. of liquid at the end of the titration.

At the beginning, the solution contained $0{\cdot}02 \times 0{\cdot}25 = 0{\cdot}005$ millimols $NaHCO_3$; at the end, $0{\cdot}000275 \times 3 = 0{\cdot}000825$ millimols, *i.e.* 16·5 % of the bicarbonate is untitrated.

Pursuing the same reasoning; supposing the titration were carried to a p_H 7·07, the millimols of bicarbonate left at the end would be 0·00045, or 9 % is untitrated.

As, when serum is substituted for pure $NaHCO_3$ solution, the value obtained agrees with the gasometric when the titration is carried to p_H 7·07, some compensation must occur. In serum, leaving aside the NaCl and the small amount of phosphate, the basic ions are balanced by the ions of two weak acids, H_2CO_3 and protein. The amount of base balanced by the latter depends on the hydrogen ion concentration. If titration is taken to p_H 4·7, the iso-electric point of serum albumin, the whole of this base can be titrated. If the titration is taken to a c_H short of this only a proportion of the base will be titrated. It would appear that when acid is added until the $p_H = 7{\cdot}07$, the amount of base balanced by protein which is titrated about equals the amount of bicarbonate untitratable.

To ascertain whether varying the p_H by about 0·3, *i.e.* doubling the c_H, would increase the base balanced by HCO_3' at the expense of that balanced by protein anions sufficiently to effect the above compensation, we determined the "alkali reserve" of a serum gasometrically, according to the method of Van Slyke, firstly when the serum was brought into equilibrium with 41·7 mm. pressure of CO_2 and secondly with 86·6 mm. pressure of CO_2. In the first case the volume CO_2 % (as bicarbonate) obtained was 50·2, in the second 55·1.

The bicarbonate in the serum was therefore $0{\cdot}0223M$ and $0{\cdot}0245M$ respectively; an increase of 10 %. The pressure of CO_2 was 2·08 times as great in the second as in the first of our experiments. This would diminish the p_H by 0·32, approximately the difference between 7·4 and 7·07. It appears, therefore, that, under the conditions obtaining in our method, the bicarbonate untitratable in the presence of air containing 0·05 % CO_2 and the amount of protein base titrated by changing the end-point 0·3 p_H are of the same order of magnitude. We conclude, therefore, that the compensation is brought about in this way.

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* $a^{18^\circ}\ CO_2$ is coefficient of absorption of CO_2 in water at 18°.

About 9 % of the bicarbonate is untitrated when the air contains 0·05 % CO_2. If the amount of CO_2 in the air of the room increases, the untitratable bicarbonate increases but the amount of protein base titrated does not vary. Consequently, a lower value is found. This is quite obvious when working in an ill-ventilated laboratory; in fact, variation in the CO_2 of the air is the chief source of error inherent in the method.

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LXXXVI. THE BICARBONATE OF THE PLASMA AND THE HYDROGEN ION CONCENTRATION OF THE BLOOD OF GUINEA-PIGS SUFFERING FROM SCURVY.

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In 1900 Wright examined the blood of six patients suffering from scurvy and found a decrease in the alkalinity of the blood. Some of these cases improved rapidly subsequent to treatment with alkalies [1900]. He obtained similar results with infantile scurvy [1908]. From these observations Wright concluded that scurvy was caused by a condition of acidosis brought about by a diet deficient in organic salts of sodium and potassium. Lamb [1902] examined scorbutic prisoners in an Indian jail, but was unable to detect any decrease in the alkalinity of their blood. The prisoners did not improve after the administration of alkalies.

Hess and Killian [1918] estimated the bicarbonate in the blood of a number of cases of infantile scurvy by Van Slyke's method and found somewhat less than the normal amount. The amount was represented by 40–45 vols. % CO_2.

Evidence of alteration in c_H is lacking.

Some diminution in the quantity of bicarbonates in scurvy is not unlikely for a scorbutic diet is generally one which affords an acid ash.

The hypothesis that acidosis is the cause of scurvy was not in accordance with early observations. It was apparent 200 years ago that fresh vegetables and fruits, especially those of the citrus variety, readily prevented and cured scurvy, whereas dried vegetables were ineffective. The organic salts were presumably still present after drying.

Cockburn (1696) concluded "that no moisture whatever could restore the natural juices of the plant lost by evaporation and altered by a fermentation which they underwent in drying." Lind [1772] quotes instances in which dried vegetable foods had been found to be useless in the treatment of scurvy. The work of Holst and Frölich on the experimental production of scurvy in guinea-pigs greatly facilitated the study of its pathology and enabled the antiscorbutic value of different food stuffs to be biologically assayed [1907, 1908]. Latterly, Zilva [1924] has concentrated the active fraction of lemon juice and found that less than half a milligram daily was sufficient to protect guinea-pigs fed on a diet which otherwise led to fatal scurvy. The very minute amount of the substance necessary to bring about this anti-scorbutic effect completes the evidence of its specific properties.

A scurvy-producing diet is, as Wright pointed out, generally one which on combustion affords insufficient base and it seemed worth while to find out whether experimentally produced scurvy in guinea-pigs is always associated with a diminution in the bicarbonate in the blood and whether any alteration in the reaction of the blood occurred.

EXPERIMENTAL METHODS.

The blood was obtained by cardiac puncture and collected under paraffin in centrifuge tubes containing a little powdered potassium oxalate.

The bicarbonate of the plasma was titrated by the micro-method described in the foregoing paper [Lepper and Martin, 1925]. The results are expressed as vols. CO_2 %, because most workers are in the habit of thinking of "alkali reserve" in these terms.

This method we found to agree with the gasometric method of Van Slyke to within ± 5 %.

The hydrogen ion concentration of the blood was estimated by the method of Dale and Evans [1920]. The dialysis was carried out at room temperature which varied from 16–22°. The temperature of the dialysate varied from 18–22°.

The collodion sacs used required 3 cc. of blood to fill them, but it was found immaterial whether this amount of blood was used or 1 cc. introduced under oxalated saline. Only the smaller amount of blood was available in some cases.

We have not adjusted our observations of p_H at room temperature to the corresponding values for blood at 38° as the amount to be subtracted is still uncertain, nor has a correction for the effect of variation of temperature on the standard phosphates been made as this would be within the error of our observation.

We are indebted to Prof. Martin for the following note on the correction for the effect of temperature on the dialysate:

"The p_H of the corresponding blood at 38° would be about 0·14 lower than the figures ascribed to the dialysates, if we assume that Hasselbalch's observation, that ox blood at 38° has the same p_H as at 18° when in equilibrium with the same pressure of CO_2, is applicable to human blood. To produce Hasselbalch's result the diminished acidity due to the lesser CO_2 concentration in the liquid at the higher temperature must be compensated by the increase in the dissociation constants of the weak acids, H_2CO_3, serum protein and oxyhaemoglobin.

Raising the temperature from 18–38° diminishes the concentration of CO_2 in water which is in equilibrium with CO_2 at constant pressure to 0·59 of the original amount. The result of this on the $\frac{CO_2}{\text{Bicarb.}}$ ratio would raise the p_H of a bicarbonate solution about 0·23. The effect on blood would be less owing to the buffering action of the serum proteins and haemoglobin. According to Hasselbalch's [1916, p. 126, Fig. 4], and Parson's [1920, p. 346, Fig. 1], observations on human blood, the rise would be 0·13 supposing the pressure of CO_2 were 40 mm. As the p_H was found to be the same at 18° and 38° with pressure of CO_2 constant, the increase in the dissociation constants of some or all of the weak acids with rise in temperature must produce an opposite effect of the same magnitude.

The effect of temperature upon the K of serum protein and haemoglobin has not, as far as I am aware, been determined, but the dissociation constant of the first H^+ in H_2CO_3 does indeed increase by this amount on raising the temperature from 18–38°, p_K at 38° being 0·13 less than at 18°.

In Dale and Evans' technique, loss of CO_2 is avoided as much as possible and only the effect of a rise in K is manifested.

It is by no means certain, however, that Hasselbalch's observations on ox blood are quantitatively applicable to human blood, for Cullen found a considerable variation in the differences in p_H at 20° and 38° in the case of horse, dog and human plasma and even between plasma and serum of the same animal."

The diet given to each control guinea-pig was about 60 g. of cabbage, 40 g. of hay and 95 g. of mixed oats and bran per day.

The diet of the guinea-pigs on which scurvy was produced consisted of oats and bran (two volumes of bran to one of oats) and about 40 cc. of autoclaved milk. On this diet the animals developed scurvy after about 15–21 days and succumbed to the disease after a month.

We have examined guinea-pigs (250–300 g.) which have subsisted on such a scorbutic diet for 10–25 days and have assessed the severity of the disease as "slight" when only a few haemorrhages were observed, as "moderate" when definite enlargement of the costochondral junctions, moderately haemorrhagic zones and brittleness of bones occurred, and as "severe" when the haemorrhages, the enlargement of the costochondral junctions, and the brittleness of the bones were very marked.

I. THE BICARBONATE OF THE PLASMA OF NORMAL GUINEA-PIGS.

The results are shown in Table I.

It will be seen that there is a large difference in the bicarbonate content between the extreme observations both for blood drawn from the right and from the left heart. For arterial blood this amounted to as much as 33 vols. %, for venous 32 vols. %. Such wide variations in bicarbonate have not been observed in man even in diabetes.

Table I. *The bicarbonate of the plasma of normal guinea-pigs.*

Number of guinea-pig	Vols. % CO_2 in plasma from left heart	Number of guinea-pig	Vols. % CO_2 in plasma from right heart
1	37·0	17	36·0
2	23·25	18	60·5
3	56·5	19	65·25
4	45·7	20	48·5
5	29·5	21	61·0
6	50·0	22	65·75
7	25·4	23	68·25
8	48·25	24	56·25
9	48·0	25	57·5
10	48·0	26	53·5
11	28·5	27	48·0
12	42·0 38·0		
13	38·0		
14	42·75		
15	49·75		
16	34·75		
Total 17	Arterial plasma, mean 40·3 vols. %	Total 11	Venous plasma, mean 56·4 vols. %

Observations by Cullen and Howard Robinson [1923] on human normal venous plasma showed extremes of 59·6 and 71 vols. %, a difference of only 11·9 %. The venous blood was drawn from the arm after a period of rest and with precautions against stasis. Buell [1919] found that in guinea-pigs the bicarbonate was low when the animal struggled during the experiment. No such relation could be found in our observations. The guinea-pigs were firmly held by a skilled assistant and the movements of the animal were limited and of short duration.

The values obtained when "slight struggling" was noted were indeed about 8 % higher for both arterial and venous blood. In three guinea-pigs in which "considerable struggling" occurred the results were, venous blood 61 vols. %, arterial blood 28·5 and 34·75 vols. % respectively.

As such wide variations were found in these different guinea-pigs, samples of arterial and venous blood were collected from the same animal. The results are shown in Table II.

Table II. *The bicarbonate of the plasma of arterial and venous blood of the same guinea-pig.*

No. of animal	Vols. % CO_2 in plasma from left heart	Vols. % CO_2 in plasma from right heart
1	48·0	68·25
2	28·5	57·5
3	42·75	53·5
4	34·75	48·0

The arterial and venous blood of the guinea-pigs collected under the conditions of our experiment showed a difference in the bicarbonate content of 10–20 vols. % CO_2. It is probable that the relation to food and the stage of digestion reached in the alimentary tract is of importance, but unfortunately no special precautions were taken to control this factor as its possible influence was not realised at first. In almost all cases the blood was collected before the animals had been fed, but no note was made as to whether food remained in the cage, so that some animals may have eaten much more recently than others.

II. THE BICARBONATE OF THE PLASMA OF GUINEA-PIGS SUFFERING FROM SCURVY.

The results are shown in Table III.

Table III. *The bicarbonate of the plasma of scorbutic guinea-pigs.*

Number of guinea-pig	Vols. % CO_2 in plasma from left heart	Degree of scurvy	Number of guinea-pig	Vols. % CO_2 in plasma from right heart	Degree of scurvy
1	27·5	Slight	18	44·0	Slight
2	29·0	,,	19	56·75	,,
3	38·5	,,	20	45·5	Severe
4	42·5	,,	21	50·0	,,
5	33·25	,,	22	55·5	,,
6	33·0	,,			
7	29·5	,,			
8	42·0	,,			
9	39·0	,,			
10	39·75	Moderate			
11	38·0	,,			
12	32·25	,,			
13	36·0	,,			
14	40·0	,,			
15	37·5	Severe			
16	32·25	,,			
17	37·5	,,			
Total 17	Arterial plasma, mean 36 vols.%		Total 5	Venous plasma, mean 50·3 vols.%	

It will be seen that the scorbutic guinea-pigs had on the average a slightly lower amount of bicarbonate in both arterial and venous plasma than the normal animals, the difference being about 5 vols. % CO_2. This difference is much less than that between the extreme values found in normal animals and might be considered to be without significance, were it not for the fact that the bicarbonate of all these scorbutic guinea-pigs is consistently low.

In order therefore to find out whether this slight alteration in the "alkali reserve" was due to the disease or to a deficiency of sodium and potassium salts in the diet, a number of guinea-pigs were put on a scurvy-producing diet with the addition of 0·8 g. of sodium citrate. The citrate was given by the mouth through a tube daily but was omitted on the day the blood was taken except in three cases recorded in Table V which will be referred to later.

The animals developed well-marked scurvy.

The results are shown in Table IV.

It will be seen that the bicarbonate of the plasma of scorbutic guinea-pigs 24 hours after the dose of citrate is still on the average slightly less than that of normal animals, the variation from the mean is however much greater than that found in scorbutic animals which are not having citrate and approximates to the normal variation.

Table IV. *The bicarbonate of the plasma of scorbutic guinea-pigs 24 hours after the last dose of sodium citrate.*

Number of guinea-pig	Vols. % CO_2 in plasma from left heart	Vols. % CO_2 in plasma from right heart	Degree of scurvy.
1	32·5	47·0	Slight
2	45·5	55·5	,,
3	22·7	54·5	Severe
4	39·75	—	,,
5	34·0	—	,,
6	42·75	—	,,
7	50·75	—	,,
Total 7	% CO_2 arterial plasma 38·2	% CO_2 venous plasma 51·3	

Table V. *The bicarbonate of the plasma of scorbutic guinea-pigs 3 hours after the last dose of sodium citrate.*

Number of guinea-pig	Vols. % CO_2 in blood from left heart	Vols. % CO_2 in blood from right heart	Degree of scurvy
1	52·5	66·5	Slight
2	55·5	—	,,
3	52·25	86·5	,,
Total 3	% CO_2 arterial plasma 53·46 (normal 40·7)	% CO_2 venous plasma 76 (normal 56·3)	

Table V shows that if the blood of the scorbutic guinea-pigs is collected 3 hours after the last dose of citrate then the bicarbonate content of both the arterial and venous blood is higher than the normal average.

These observations indicate that:

1. The low mean level of the bicarbonate in the blood of scorbutic guinea-pigs is associated with a deficiency of sodium and potassium salts of organic acids in the scurvy-producing diet.

2. The addition of sodium citrate to the diet restored the normal bicarbonate content of the blood, but did not influence the onset of scurvy.

III. THE HYDROGEN ION CONCENTRATION OF THE BLOOD OF GUINEA-PIGS SUFFERING FROM SCURVY.

The p_H found in the blood of normal guinea-pigs is shown in Table VI. The two chief experimental errors in Dale and Evans' method are:

1. Loss of CO_2 from the blood during dialysis.
2. Access of CO_2 to the standard phosphate solutions.

Care was taken to avoid these sources of error as far as possible but as they both give too high p_H readings, observations made by this method tend to be too alkaline rather than too acid.

The p_{H} of the blood at 38° would probably be about 0·15 lower than that of the dialysates at room temperature (see Prof. Martin's note on p. 582).

Table VI. *The* $\mathrm{p_H}$ *of the dialysate of the blood of normal guinea-pigs. Observations at room temperature.*

Number of guinea-pig	p_{H} of blood from left heart	p_{H} of blood from right heart
1	7·61	—
2	7·61	—
3	7·645	—
4	7·58	—
5	—	7·46
6	7·57	—
7	7·59	—
8	—	7·53
9	7·56	—
10	7·6	—
11	7·62	—
	Total 9. Arterial blood, mean 7·59	Total 2. Venous blood, mean 7·49

The blood was taken before the animals had been fed and no marked struggling was noted in any case.

The scorbutic animals were examined under similar conditions. Samples of blood were taken between the 15th and 24th day after the diet was started. One of the guinea-pigs was killed at each experiment and examined post-mortem so that an estimate of the degree of scurvy present could be arrived at; the other guinea-pigs were kept alive and re-examined some days later when the scorbutic condition had become very severe.

The results are shown in Table VII.

Table VII. *The* $\mathrm{p_H}$ *of the dialysate of the blood of guinea-pigs suffering from scurvy. Observations at room temperature.*

No. of guinea-pig	No. of days on scorbutic diet	p_{H} of blood from left heart	p_{H} of blood from right heart	Degree of scurvy
1	15	—	7·54	
2	15	—	7·44	Slight
3	18	7·55	—	
4	18	—	7·56	Severe
5	18	7·67	—	
6	20	—	7·53	
7	20	7·65	—	
8	20	7·52	—	Moderate
9	20	7·64	—	
10	22	—	7·59	Moderate
1	22	7·51	—	Slight
3	24	7·67	—	Severe
6	24	7·64	—	,,
7	24	7·58	—	,,
9	23	7·71	—	,,
		Total 10. Arterial blood, mean 7·61	Total 5. Venous blood, mean 7·53	

No evidence of an increased hydrogen ion concentration of the blood was found in any of the guinea-pigs.

SUMMARY AND CONCLUSIONS.

There are large variations in the amount of bicarbonate in the plasma of normal guinea-pigs; the hydrogen ion concentration, on the other hand, shows only slight variations.

The titratable alkali of the plasma of guinea-pigs suffering from scurvy is on the average at a lower level than that of normal guinea-pigs. This is due to the deficiency of sodium and potassium salts of organic acids in the scurvy-producing diet. The addition of sodium citrate to the diet restores the normal bicarbonate content of the plasma but does not influence the onset of scurvy.

An increase in the hydrogen ion concentration of the blood of guinea-pigs suffering from scurvy was not observed.

We take this opportunity of expressing our thanks to Prof. C. J. Martin for much helpful criticism.

We are also indebted to Dr Mary Cowan for her skilled assistance in obtaining samples of blood from the scorbutic guinea-pigs.

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LXXXVII. THE ANTISCORBUTIC FRACTION OF LEMON JUICE. III.

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In this communication is brought forward further information in connection with the fractionation of the antiscorbutic factor and the chemical properties of active fractions. It was previously shown [Zilva, 1924] that basic lead acetate precipitated the active principle from lemon juice quantitatively and that very active fractions could be obtained in this way by removing the sugar in the decitrated lemon juice by fermentation previous to precipitation. An endeavour was made, so far without success, to find a precipitating reagent which would have the advantage over basic lead acetate of removing the factor from active solutions accompanied by less extraneous matter. In this unsuccessful quest one reagent, namely, neutral lead acetate, yielded results which are of interest and worth recording. Whilst basic lead acetate precipitates the antiscorbutic factor from decitrated lemon juice quantitatively, the neutral salt does not precipitate it at all. It, however, removes considerable quantities of non-active matter from such solutions and also from the ultimate basic lead acetate fraction described in the preceding communication of this series. This reagent is, therefore, of value in the chemical fractionation of antiscorbutic solutions. Bezssonoff [1922, 1] has also found that it does not remove the vitamin from cabbage juice.

Three experiments were instituted with the object of studying the action of neutral lead acetate on active solutions. In the first experiment decitrated lemon juice was treated with neutral lead acetate until no more precipitate was formed. The solution was then centrifuged and the precipitate dissolved in the least quantity of acetic acid. Magnesium sulphate was added to this solution until all the lead was precipitated, after which treatment two volumes of absolute alcohol were introduced and the alcoholic solution allowed to stand for about an hour. It was then filtered and the filtrate evaporated to a small volume under reduced pressure at 50°. This fraction was finally made up to the original volume of the lemon juice from which it was prepared and tested out for its antiscorbutic potency on guinea-pigs in the usual way. The doses were prepared daily except during the week-ends when 2 days' supply was prepared, acidified with citric acid, exhausted and kept in a vacuum until required for feeding. Thus the chance of deterioration on storage was reduced to a minimum. Doses equivalent to 1·5 cc., 3 cc. and 5 cc. of the original lemon juice were tested out, three guinea-pigs being employed for each dose. All the animals succumbed to scurvy within about a month.

Before it could be definitely taken that the neutral lead acetate failed to precipitate the vitamin, the presence of the latter in the solution after precipitation had to be demonstrated. With this end in view, in the second experiment, decitrated lemon juice was precipitated with neutral lead acetate, centrifuged and the resulting supernatant solution treated with magnesium sulphate and alcohol in order to remove any excess of lead. After distilling off the alcohol under reduced pressure and making up to the original volume the preparation was tested out for the antiscorbutic vitamin. Doses equivalent to 1·5 cc., 3 cc. and 5 cc. were employed in this test. Three guinea-pigs were used for each dose. The three animals on the lowest dose died of scurvy after 50 days, 34 days and 37 days respectively. Those on the 3 cc. dose died after 42 days, 44 days and 60 days. The animals on the highest dose survived for 60 days after which time they were chloroformed. Two of these animals showed very slight signs of scurvy at the post-mortem examination. The third animal was found normal.

In the third experiment 1500 cc. of decitrated lemon juice were concentrated to 600 cc. and precipitated with neutral lead acetate. The precipitate was centrifuged quickly and the supernatant fluid treated with an excess of basic lead acetate. A precipitate was formed which was centrifuged and dissolved in acetic acid. The acid solution was precipitated with magnesium sulphate and by the subsequent addition of two volumes of absolute alcohol and filtered. The alcohol from the filtrate was removed by distillation under reduced pressure and the volume was eventually made up to 1500 cc. This batch was acidified with citric acid and stored in a vacuum. A sufficient quantity was withdrawn every day for 6 weeks for testing purposes. It was found almost unworkable to prepare daily fresh doses for testing purposes owing to the great labour involved. This preparation, as was to be expected, was found to be active, although a very significant loss in the antiscorbutic potency was recorded. A daily dose equivalent to 2 cc. of lemon juice failed even to delay the onset of scurvy in guinea-pigs whilst on a 3 cc. dose one guinea-pig succumbed to scurvy after 40 days and another animal after 54 days.

The above experiments make it plain that neutral lead acetate does not precipitate the active principle at all and that antiscorbutic solutions precipitated by this reagent retain their activity in the filtrate. Although in the above experiments an appreciable loss was observed after precipitation with neutral lead acetate, there is no doubt that it is brought about by the general manipulation and not by the precipitating agent. Experiments to be described below corroborate this assumption.

The action of neutral and of basic lead acetate on the antiscorbutic factor in another source, namely, swede juice, was next investigated. In this case too the basic salt precipitated the active principle whilst the neutral did not.

In one experiment the root was expressed and the turbid juice thus obtained was treated with neutral lead acetate until no more precipitate was formed. After centrifuging, the excess of the lead salt in the supernatant fluid was removed with magnesium sulphate and alcohol as described before. After distilling off the alcohol, the preparation was made up to its original volume. The dry matter estimated on seven different samples of this preparation varied from 4·4–6·8 %, giving an average of 5·1 %. Daily doses of 3 cc. were given to three guinea-pigs and of 5 cc. to two animals. Owing to the intervention of the Christmas holiday this set of animals was chloroformed 53 days instead of 60 days after the commencement of the experiment. The guinea-pigs which received the 3 cc. dose showed very mild scurvy at the post-mortem examination. The animals receiving the higher dose did not show any signs of scurvy at all. There was no doubt that both sets of animals could have survived the usual 60 days. As the minimum protective dose of unprecipitated swede juice was found to be 3 cc. it is safe to conclude that the neutral lead acetate did not precipitate any of the active principle at all. The experiment also shows that the loss incurred in a similar experiment with lemon juice mentioned above was not due to the precipitating agent.

In another experiment the swede juice was treated with two volumes of alcohol and filtered. After removing the alcohol by distillation at a low pressure the solution was made up with distilled water to its original volume. This treatment removes a good deal of non-active matter which is precipitated by basic lead acetate. After treatment the solution was precipitated with basic lead acetate and the precipitate after centrifuging was taken up in acetic acid. Perfect solution could not be effected in this way. Without filtering, however, the solution was treated with magnesium sulphate and then with alcohol as described before in order to remove the lead; after distilling off the alcohol it was made up to the original volume of the swede juice. The dry matter estimated on seven specimens varied from 1·1–2·8 % with an average of 1·5 %. There was, therefore, a considerable reduction in the total solids as compared with the swede juice preparation of the previous experiment. This was mainly due to the reduction of the sugar content. Five guinea-pigs were used in this experiment, three on a daily dose equivalent to 3 cc. of the original juice and two on a daily dose of 5 cc. All the animals as in the preceding experiment had to be chloroformed after 53 days and none showed any scorbutic signs at the post-mortem examination.

The behaviour of the antiscorbutic factor in swede juice towards these two precipitating reagents is, therefore, the same as in lemon juice.

In a previous communication it was pointed out that the purest fractions showed a very low nitrogen content. An attempt was made to study the chemical nature of the nitrogenous constituents of such fractions. On careful examination they were found to contain both amino and amide nitrogen. The latter is small in amount and escaped detection at first owing to the fact that too little of the preparation was employed in the test. The presence of amide nitrogen can, however, be demonstrated in the following way. Decitrated lemon juice equivalent to 200 cc. of lemon juice is first precipitated with basic lead acetate, the precipitate suspended in distilled water and decomposed with hydrogen sulphide and the hydrogen sulphide removed. The solution is then precipitated with neutral lead acetate. filtered, and the filtrate treated with hydrogen sulphide, concentrated, precipitated with absolute alcohol and the filtrate eventually made up to 50 cc. To this 5 cc. of concentrated sulphuric acid are added and the mixture is autoclaved at 5–10 lbs. pressure for 2 hours. It is then distilled with 40 cc. of 40 % NaOH and the ammonia received in $N/50$ sulphuric acid. Amide nitrogen equivalent to 1–2 cc. $N/50$ ammonia per 200 cc. of original lemon juice can be demonstrated in every case.

The amino nitrogen was estimated in a Van Slyke micro-apparatus. The following were the quantities of the reagents employed. 2·5 cc. glacial acetic acid, 10 cc. of nitrite solution and 3 cc. of a preparation equivalent to 60 cc. of the original solution obtained as described above. In the blank estimation 3 cc. of distilled water replaced the concentrated preparation. About 80 % of the gas was given off in the first 15 minutes and the entire evolution of the gas was usually complete in 2–4 hours. Quantities of amino nitrogen of the order of 0·5 cc. were thus obtained for the equivalent of 60 cc. of lemon juice.

The sum of the amino and amide nitrogen, however, forms only a part of the total nitrogen as will be seen from the figures obtained from three representative batches. The equivalent of 200 cc. of original juice was used in each estimation.

	Batch I	Batch II	Batch III
	%	%	%
Total nitrogen	0·001386	0·00175	0·00119
Amino ,,	0·0003693	0·000554	0·0004683
Amide ,,	0·0001680	0·000196	0·00014
Percentage of amino and amide nitrogen	38·7	42·85	51·11

It was possible to show that the amide group was not indispensable for the antiscorbutic activity. 50 cc. of concentrated lemon juice were treated with 5 cc. of concentrated H_2SO_4. The solution, after being exhausted under a vacuum pump, was saturated with carbon dioxide and the receptacle filled with that gas. It was then placed in an autoclave, which was brought up to 5–10 lbs. pressure, after which it was cooled down. Preliminary experiments had shown that this treatment was sufficient to remove all the amide nitrogen. Before testing, the solution was neutralised with calcium carbonate and filtered. With the exception of the week-end this preparation was carried out daily for testing purposes. Of the eight guinea-pigs employed in this test the three receiving 1·5 cc. per day succumbed to scurvy in the usual time, of those receiving 3 cc. per day two lived for 46 days and 48 days respectively, whilst one survived for 60 days. The two animals on the 5 cc. dose lived for 60 days, after which time they were chloroformed. One of these animals showed slight, the other no scorbutic signs at the post-mortem. It is thus seen that although the drastic treatment destroyed some of the activity the solution was markedly active, notwithstanding that all the amide nitrogen had been removed.

It is more difficult to ascertain whether the amino nitrogen has any bearing on the activity. The difficulty of feeding deaminated products without causing any ill-effects to the animals has not yet been overcome. The formation of double compounds with reagents that usually combine with amino acids, amino purines and similar compounds has also met with little success so far and the problem is still under investigation. The study of the chemical nature of the concentrated fractions is being continued. The great obstacle in the way of chemical progress at this stage is the fact that the concentrated fractions are of a syrupy consistency and consequently are not very amenable to separation and identification. By repeated precipitation with alcohol crystals are sometimes obtained.

Bezssonoff in a recent investigation [1925] utilising a scheme of fractionation similar to mine [Zilva, 1924] on cabbage juice, namely, the repeated precipitation by basic lead acetate and alcohol, has also obtained crystals by extracting such a fraction with acetone and found them to be active. The animal tests for activity employed by Bezssonoff are, however, in the author's opinion, entirely unsatisfactory, and in any case the great likelihood must be borne in mind that the activity of a crystalline fraction may not be due to the crystalline substance itself. This is evident from the work done by various investigators on the fractionation of vitamin B.

It is of interest to note that Bezssonoff finds that the above fraction does not give a coloration with the phosphomolybdotungstic acid reagent suggested by him for the detection of the antiscorbutic vitamin. This would not be surprising even if the activity of this fraction were confirmed, since it was found [Connell and Zilva, 1924] that the active concentrated fraction previously obtained by the writer failed to react with the reagent [cf. Bezssonoff, 1922, 2; 1923; Kay and Zilva, 1923; Bezssonoff, 1924].

SUMMARY.

Unlike basic lead acetate, which precipitates the antiscorbutic factor from lemon juice quantitatively, neutral lead acetate does not precipitate it at all.

The behaviour of these two precipitating reagents towards the antiscorbutic factor in swede juice is the same as to that in lemon juice.

Small quantities of amino and amide nitrogen are present in certain concentrated antiscorbutic fractions. They, however, form only a part of the total nitrogen content which is of the order of 1–2 mg. %.

There is no connection between the amide nitrogen and the antiscorbutic activity of lemon juice.

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LXXXVIII. THE EFFECTS OF PARATHYROID FEEDING ON CALCIUM AND CREATINE METABOLISM.

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It has been known for some years that removal of the parathyroid glands results in a condition of tetany. A large number of observations on the nervous and metabolic changes in tetania parathyreopriva have been recorded, and from these experiments conclusions have been drawn as to the probable function of these glands and two main hypotheses advanced to explain the effect of their removal. The first theory associates the nervous changes with a deficiency of calcium, and the second attributes them to the production of a toxic agent, possibly guanidine, which is closely related to creatine.

The experiments of MacCallum and Voegtlin [1909] and MacCallum and Vogel [1913] have indicated that the parathyroids are associated with the retention of calcium. Korenchevsky [1922] finds that no change in the calcium content of bone results on removal of the parathyroids. Vines [1921, 1922] has applied the use of parathyroid extracts to the treatment of certain diseases which he associates with a diminution in the ionic calcium of the blood, and by feeding parathyroid he was able to raise the ionised calcium to the normal value.

Collip, Clark and Scott [1925] report that parathyroid extracts are of great benefit in removing the tetany of parathyroidectomised dogs and that administration of potent extracts to normal dogs results in hypercalcaemia.

Paton and Findlay [1916] have indicated that the symptoms of parathyroidectomy are due to the liberation of methylguanidine. Frank, Stern and Nothman [1921] suggest that the toxic agent is dimethylguanidine. Both these substances are closely related to creatine and creatinine. An increase in the excretion of creatine after parathyroidectomy has been found by Greenwald [1911] and a decrease in creatinine by Burns [1916] and by Greenwald. Henderson [1918] has shown that tetania parathyreopriva is associated with an increase in muscle creatine and a decrease in the total and free guanidine of muscle. Hammett [1921, 1] has demonstrated a direct relationship between the parathyroids and creatine metabolism. He found that the addition of parathyroid tissue to muscle extracts retarded the formation of creatinine which normally occurs during incubation.

The present investigation was started with the object of ascertaining the effect of parathyroid feeding and injections on the creatine metabolism of rats, as measured by muscle creatine and urinary creatine and creatinine. It was later extended, and a further series of experiments was carried out to determine the action of parathyroid feeding on calcium metabolism with reference to bone calcium.

The thyroid and parathyroid glands of several rats were examined for histological changes. The results of this part of the work will be reported later.

In every experiment the rats were weighed twice a week so that variations in weight and growth could be followed, and in all experiments except the injection series the food was weighed out and the residues collected daily and weighed in order to determine the effect of parathyroid feeding on appetite.

METHODS.

Feeding. Adult and young rats of both sexes were used for these experiments. They were fed on a diet of bread, butter and water. The desiccated parathyroid, in doses of 0·02 g. or 0·1 g., was mixed with a little bread and water and given to the rats before their main meal. No further food was given until all the parathyroid had been eaten. The rats were fed at approximately the same time each afternoon and the food removed early next morning. The parathyroid was administered daily for periods of 2 or 4 weeks.

Injections. The parathyroid extract was injected daily for 4 weeks in doses of 0·5 cc. (= 0·00085 g. desiccated gland).

In another series of experiments calcium lactate (0·21 g.) was given daily with and without parathyroid.

Estimations. Muscle Creatine. The total creatine and creatinine of muscle was estimated as creatinine by the method of Janney and Blatherwick [1915]. The right hind limb was freed from muscle, as far as possible without injury to the blood vessels to avoid errors due to adherent blood. The muscle was well minced and a portion (3·5 g.) used for the creatine estimation; the other portion was used to determine the water content.

Urinary Creatine and Creatinine. This series of experiments was carried out on four rats which were kept in small metabolism cages. In the experiments on the first three rats, 48-hourly samples of urine were collected for a period of 4 weeks. During the first 2 weeks the rats were fed on bread and butter alone, and during the second 2 weeks they received 0·02 g. parathyroid daily in addition. In the case of the fourth rat 24-hourly samples of urine were collected. The rat received bread and butter for the first 8 days, then 0·02 g. parathyroid was added daily to the diet for 11 days. The urine was collected in a flask containing a trace of thymol, and was made up, together with the washings of the floor of the cage, to constant volume as recommended by Burns and Orr [1914]. Estimations were done on 24-hourly samples of urine + thymol to determine if any change in creatine-creatinine content takes place if urine is kept for a day before estimating. The results showed that no appreciable change occurs.

The creatine and creatinine were estimated by the micro-method of Folin [1914] using a standard of 0·2 mg. creatinine per cc. which was made up fresh for each estimation. Picric acid, purified and tested by the method of Folin and Doisy [1916] was used throughout the experiments. In order to correct any personal errors in reading the colorimeter, curves were plotted similar to those reported by Mellanby [1908] and by Hunter and Campbell [1916]. The urine was tested throughout the experiments for acetone bodies, but none was found.

Bone Calcium. The bones used in every case were the right fibula, tibia and femur. The fibula and tibia were separated from the femur and all three carefully freed from muscle, cartilage, etc. After drying at 107° for 16–17 hours to determine moisture content, the calcium was estimated in the dried bone by Aron's [1908] method.

RESULTS.

Appetite and Weight. Observations were made on 118 rats but no definite relationship between appetite and weight was recorded. There appeared to be a slight increase in the appetite of the rats which received parathyroid; this effect of parathyroid feeding has also been observed by Kojima [1917]. In the young rats the variations in weight between the controls and those receiving parathyroid were too small for it to be concluded that parathyroid feeding has any effect on growth, confirming the results of Cameron and Carmichael [1921].

Muscle Creatine. The results for the average muscle creatine values for the different series are given in Table I. In the results for the adult rats (series 1–6) the only marked difference found is in series 4, and this was due to one of the rats giving an abnormally high value (0·546), so that it may be concluded that parathyroid feeding has no effect on muscle creatine.

Table I.

Series	No. of rats	g. creatine per 100 g. moist muscle	g. creatine per 100 g. dry muscle	g. moisture per 100 g. muscle	Diet daily g.	Mode and duration of administration
Adult Rats.						
1	21	0·478	1·923	75·2	*N*	—
2	18	0·483	1·877	74·4	0·02 *P*	Orally—2 weeks
3	8	0·476	1·993	76·0	0·02 *P*	,, 4 ,,
4	3	0·507	2·022	74·8	0·1 *P*	,, 4 ,,
5	6	0·481	1·843	73·3	0·21 Ca lactate	,, 2 ,,
6	7	0·475	1·879	74·6	0·21 Ca lactate 0·02 *P*	,, 2 ,,
Young Rats.						
7	4	0·396	1·496	73·4	*N*	,, 2 ,,
	4	0·419	1·633	74·4	0·02 *P*	
8	4	0·459	1·836	74·9	*N*	,, 2 ,,
	4	0·468	1·832	74·5	0·02 *P*	

N=Normal diet. *P*=Parathyroid.

The results for the young rats confirm those of previous observers that there is less creatine in the muscle of young rats than in that of adult rats. In both series a slightly higher creatine value for moist muscle was found in the rats which received parathyroid.

Urinary Creatine and Creatinine. The results of the urine estimations are given in Table II. The results are expressed as the average for the period of time mentioned. The normal average creatinine coefficient of rats 1, 3 and 4 is 13·5. Rat 2 is omitted from the average as it is apparently an exception showing a very high creatinine coefficient (19·5) which is associated with an abnormally high creatinine excretion in proportion to its weight. The normal average creatine of rat's muscle is given in Table I as 0·478 g. per 100 g. moist muscle. These results, taken in conjunction with those of Myers and Fine [1913], suggest that there is a relationship between the creatinine elimination and the percentage of muscle creatine of a given species, and the values obtained for the rat place it between the rabbit and man.

Table II. *Urine estimations.*

Adult Rats.

Average values only are given.

Rat	Creatinine per 2 days mg.	Creatine per 2 days mg.	Creatinine coefficient	Diet g.	Duration of experiment (days)
1 (♂)	12·9	2·8	11·4	*N*	14
	14·35	2·7	12·8	0·02 *P*	14
Difference	+1·45	−0·1	+1·4		
2 (♂)	16·54	1·9	19·5	*N*	14
	14·8	5·8	17·9	0·02 *P*	14
Difference	−1·74	+3·9	−1·6		
3 (♂)	15·33	2·37	14·5	*N*	14
	15·03	2·61	13·1	0·02 *P*	14
Difference	−0·30	+0·24	−1·4		
	Creatinine per day	Creatine per day			
4 (♀)	7·86	1·8	14·6	*N*	8
	7·6	2·2	13·9	0·02 *P*	11
Difference	−0·26	+0·4	−0·7		

N = Normal diet. *P* = Parathyroid.

Creatine in varying quantities is found to be present normally in rat's urine [Folin and Morris, 1913].

An increase in the average creatine excretion is shown by rats 2, 3 and 4. This increase is associated with a decrease in creatinine excretion, and in rats 3 and 4 might be due to the alteration in the creatine-creatinine ratio, and not to a direct effect on creatine metabolism, since these rats show practically no alteration in the amount of total creatinine excreted. Rat 2, on the other hand, shows a marked increase in the amount of total creatinine excreted and therefore suggests that parathyroid feeding not only alters the creatine-creatinine ratio in the urine, but also stimulates the production of creatine.

The results of the first experiment apparently contradict this effect. During the first 12 days on normal diet the creatinine excretions of this rat varied from 11·7–12·8 mg. per day. During the 2 days previous to the commencement of parathyroid feeding the creatinine excretion rose to 15·5 mg.—higher than the average value when receiving parathyroid. The apparent contradiction of these results, then, may be due to the fact that the value of creatinine excretion in rat 1 rose suddenly during the normal period and might have remained at this high level regardless of addition of parathyroid to the diet.

Bone Calcium. Table III shows the average values for the calcium content of the bone of rats on normal diet and of those receiving parathyroid. In the case of the adult rats the bone calcium content is very similar for all three series, whilst the very slightly higher average value found in the case of the young rats fed on parathyroid as compared with the controls does not warrant the conclusion that parathyroid feeding has any definite effect.

Table III.

Adult Rats.

Series	No. of rats	g. Ca per 100 g. moist bone	g. Ca per 100 g. dry bone	g. moisture per 100 g. bone	Diet g.	Mode and duration of administration
1	7	16·2	23·3	30·6	*N*	None
2	7	15·9	22·2	28·9	0·02 *P*	Orally—4 weeks
3	3	15·5	22·7	31·3	0·1 *P*	,, 4 ,,
Young Rats.						
4	3	11·8	19·8	39·9	*N*	Orally—4 weeks
	3	12·3	20·8	40·2	0·02 *P*	
5	5	9·71	17·3	45·9	*N*	Orally—4 weeks
	5	10·07	18·4	44·9	0·02 *P*	
6	3	9·17	17·2	45·9	*N*	Injected—4 weeks
	3	9·46	18·4	48·4	0·0008 *P*	

N = Normal diet. *P* = Parathyroid.

GENERAL DISCUSSION.

The output of guanidine after parathyroidectomy, and the close chemical relationship between creatine and guanidine, suggest that the parathyroid may be an important factor in creatine metabolism. The experiments of Hammett [1921, 2] indicate that parathyroid extract inhibits the conversion of creatine into creatinine in incubated muscle. If muscle creatine is the origin of urinary creatinine, as is believed by some observers, it would be expected, since parathyroid extract inhibits the conversion of creatine to creatinine in muscle, that feeding with parathyroid would cause a decrease in the elimination of the latter associated with an increase in that of the former. The results reported here support this view.

The negative effect of parathyroid feeding on muscle creatine itself may be partly due to the fact that muscle is normally practically saturated with creatine, and even if this substance is fed, only a trace is taken up by muscles of adult animals.

Experiments [Hammett, 1921, 2] which have been carried out on rats normally used for experimental purposes and those which are "stock" and only come into such human contact as is incident to cleaning, feeding, etc., showed that there was a marked difference in the response of the two groups to removal of the parathyroids. The majority of the animals which survived belonged to the "experimental group" and Hammett suggested that their survival was due to an inherently smaller proportion of tetany-producing substances. The rats used in the present work were not equally tractable; some were much more excitable than others and this difference in excitability, if considered with the results of Hammett, and those of Pekelharing [1911] on creatine and muscle tone, may explain variations found in muscle creatine. Where a high state of neuro-muscular tension existed a higher creatine content of muscle would be found.

SUMMARY.

1. Administration of extracts of parathyroid has no effect on the weight of adult animals or on the growth of young animals.

2. There appears to be an increase in the appetite of rats fed with parathyroid.

3. Parathyroid feeding has no effect on creatine metabolism as measured by muscle creatine.

4. Parathyroid feeding causes an alteration in the ratio of creatinine to creatine excreted, resulting in the elimination of more creatine and less creatinine.

5. Parathyroid feeding and injections have no effect on the deposition of calcium in bone.

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LXXXIX. UREA ESTIMATIONS ON SMALL QUANTITIES OF BLOOD.

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Much attention has been given recently to attempts to reduce the amount of blood required for blood urea estimations to considerably less than the volume ordinarily taken in the well-known titration method of Marshall, Cullen and Van Slyke, for, where extended clinical work has demanded a number of estimations on the same individual, it becomes an impracticable proposition to obtain large blood samples each time.

The methods of Hindmarsh and Priestley [1924], Rehberg [1925, 1] and Murray [1925] deviate from the classical method either in employing colorimetric means or special apparatus, whilst that of Thomas [1924] represents an adaptation of the old form to one-fifteenth of the ordinary amount of blood, by use of more dilute standard solutions of acid and alkali. With respect to the latter, however, it has been found [Murray, 1925]—and my own experience confirms the finding—that it fails to give consistent results. There would still seem, therefore, to be a place for a method following the lines of the long-established method of estimating blood urea, which overcomes in great measure the chief sources of possible error involved in the modification devised by Thomas.

In making clinical blood urea estimations, I was led to attempt to adapt the ordinary titration method to cases where only 0·2–0·5 cc. of blood were available, on somewhat similar lines to those described by the last-mentioned author, but could not produce such satisfactory results as he has done even when using a larger quantity of material. Not only did there enter the fallacy of titrating with dilute standard solutions in comparatively large volume [see Rehberg, 1925, 2], but the use of methyl red as indicator also presented difficulties unless due care were taken to perform the final titration in complete absence of CO_2.

While experimenting with different indicators, my attention was attracted to the suggestion to use a combination of methyl red and methylene blue [de Wesselow, 1924], and I had no difficulty in finding a mixture of the two which gave rise to none of the difficulties encountered in the use of methyl red itself. The combined indicator, made up as stated below, is extremely sensitive to small additions of $N/100$ alkali over the slight range of p_H in which it changes from purple to green, and when once just completely green shows no signs of giving a return colour. Using a microburette with a capillary jet coated on its exterior with a thin film of paraffin wax, each drop can be reduced to a volume of 0·014 cc., sufficient to produce quite a perceptible change in the indicator. The greatest possible error in the titration, apart from errors in the calibration or reading of the burette, is represented by this figure, equivalent to slightly over 2 mg. per 100 cc. of blood, where 0·2 cc. are employed for the actual estimation.

The technique incorporating the use of this indicator in order to work upon small quantities of blood and titrate in small volume is as follows.

1 cc. of 0·6 % potassium dihydrogen phosphate is placed in a flat-bottomed, stout glass tube and the blood sample (0·2–0·5 cc.) added to it from a capillary pipette, the latter being afterwards washed out with a portion of the phosphate solution. 0·2 g. of a well-mixed sample of finely ground soya bean meal is accurately weighed out and added to the contents of the tube, which are then incubated at 40° for 15 minutes. The ammonium salt is decomposed by the addition of 4 cc. of saturated potassium carbonate solution and 2 g. of solid potassium carbonate, a few drops of caprylic alcohol are then added, and the tube attached for aeration to sulphuric acid and titration tubes in the usual way. The titration tube is of resistance glass, 6″ × $\frac{5}{8}$″, and contains 5 cc. of $N/100$ sulphuric acid if 0·5 cc. of blood is available for analysis, 2 cc. diluted with 2 cc. of distilled water, if the estimation is being done on 0·2 cc. of blood, with 3–4 drops of an indicator made up of 100 cc. of 0·02 % methyl red and 30 cc. of 0·1 % methylene blue.

Aeration requires about 1 hour if conducted at a rate which will ensure with certainty that the ammonia will be completely absorbed by the small volume of acid used. $N/100$ sodium hydroxide added from a 5 cc. microburette (a 2 cc. burette, graduated to 0·02 cc., when 0·2 cc. of blood are taken) is used in the final titration.

The usual blank determination is performed in the same way as above, and its value subtracted from the blood values.

For 0·5 cc. blood (blood reading – blank reading) cc. × 60 = mg. urea per 100 cc. blood.

For 0·2 cc. blood (blood reading – blank reading) cc. × 150 = mg. urea per 100 cc. blood.

In this way the figures obtained with 0·5 cc. of blood all agree within 1 mg. per 100 cc. of blood with the values given by the ordinary method on 3 cc. (using the mixed indicator in place of methyl red for the latter). Typical figures obtained by use of 0·2 cc. of blood and an ordinary make of 2 cc. microburette are:

1. Standard urea solution.

Urea mg. per 100 cc.	Found
20·0	18·0
	19·5
39·5	36·0
	37·5
	40·5
98·7	94·5
	93·0
	94·5
	96·0

2. Blood urea.

Urea mg. per 100 cc.	Found
26·7 (by ordinary standard method)	27·0
43·0 ,, ,,	40·5
61·5 ,, ,,	60·0

Such figures may be taken as sufficiently accurate for most clinical purposes.

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XC. THE NATURE OF THE METABOLIC PROCESSES IN *ASCARIS LUMBRICOIDES*.

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(*Received June 17th, 1925.*)

Introduction.

It is generally accepted that the oxidation of part of the lactic acid produced in muscular contraction and the simultaneous conversion of the rest of the lactic acid into glycogen form an essential part of muscular metabolism. If these conclusions are correct the existence of an organism which requires no oxygen for its metabolism, and yet is supplied with muscles having a normal contractile mechanism, is unlikely, unless by some means the oxidative recovery phase is dispensed with and the lactic acid removed by diffusion into the vascular system and ultimately excreted. The latter process would be a costly one, liberating less than a tenth of the total energy in the original glycogen.

Weinland [1901], however, maintains that the intestinal worm *Ascaris lumbricoides* does not require oxygen for its metabolism and lives equally well in atmospheres of inert gases, whilst on the other hand, it is well known that this worm is capable of marked muscular movement. In explanation of the source from which the energy is derived, Weinland puts forward the suggestion that a process nearly allied to fermentation takes the place of the ordinary aerobic metabolism.

The present work was undertaken in the first place to test the accuracy of Weinland's conclusions in respect to the anaerobiosis of *Ascaris*, and in the second place if the worm proved to be truly anaerobic to investigate the nature of the muscular process involved. The investigation was not carried beyond the first stage as evidence was forthcoming which rendered doubtful the anaerobiosis of the worms, and precluded entirely the possibility of work in the absence of oxygen or its equivalent.

It seems advisable before proceeding to the description of the experimental work to recapitulate briefly the evidence on which the anaerobiosis is based.

Bunge [1884, 1890] states that various types of intestinal worms can live for periods of 5–6 days in the absence of air, but that they become dormant and refuse to move. Weinland [1901] repeated these experiments using a variety of gases, and states that the worms remain alive for periods up to 9 days quite independent of the surrounding gas, except that in carbon dioxide the life period is a little longer than the average. It is, however, unfortunate in view of Bunge's observations that Weinland gives no criterion by which he judged the worms to be alive, as some state akin to hibernation at once suggests itself. If the worms can, by remaining still, reduce their energy requirement to a very small amount, it is possible that there is a sufficient store of material capable of carrying out oxidation present in their bodies to maintain the necessary life processes for considerable periods, or that in these circumstances the energy is supplied by some purely temporary anaerobic process, which would be entirely inadequate for the normal energy requirements.

Other evidence advanced by Weinland consists of analyses of the body contents of the worms made at the beginning and the end of periods during which they were kept in 1 % sodium chloride solution; and further of analyses of the surrounding fluids at the end of these periods. By such means Weinland hoped to show what substances were metabolised by the worms, and what were the products of metabolism. The chief differences found by Weinland after a period of starvation in sodium chloride solution were a large fall in the glycogen content and a still larger rise in the water content. There were also small losses in the glucose and protein but not of the same order as in the case of the glycogen. The surrounding solution Weinland found to be acid and evil smelling. This he claimed to be due to the presence of valeric acid, but there is some doubt as to the validity of his findings owing to the inherent difficulty of his analytical methods.

The other product of metabolism identified by Weinland was carbon dioxide, and he explained the process involved by the following equation although he did not identify the hydrogen.

$$\underset{\text{glycogen}}{4C_6H_{10}O_5 . H_2O} = 9CO_2 + \underset{\text{valeric acid}}{3C_5H_{10}O_2} + 9H_2 .$$

The tentative suggestion put forward by Weinland that the hydrogen reduced some other body constituent is only another way of postulating a hydrogen acceptor, which must be used up in the process and whose absence would probably cause death.

In order to meet the criticism that acids similar to valeric (acetic, butyric, lactic and succinic) are produced by intestinal bacteria, Weinland [1902] pressed the worms and obtained a juice which he sterilised by the addition of disinfectants such as sodium fluoride, toluene, etc. This sterile juice he added to solutions of glucose and glycogen and obtained results similar to those which were given by the worms, *i.e.* valeric acid formed in the solution and carbon dioxide given off. In a more recent paper Anton Fischer [1924] does not agree with these findings. Fischer estimated the total acidity in a solution in which *Ascaris megalocephala* had lived, and also the lactic acid, and found that the lactic acid amounted to about 10 % of the total acidity. If, however, the worms were first ground up and the pulp sterilised with toluene, the only products of autolysis were lactic and phosphoric acids. In view of this work it would appear that Weinland had not succeeded in rendering the pressed-out juice free from bacteria, and hence there is no evidence that the volatile fatty acids found in connection with the worms are not the products of bacterial action.

The present work divides itself into two sections:

(1) an attempt to repeat Weinland's experiment on the length of life of *Ascaris lumbricoides* in hydrogen, but at the same time using means to ensure their continued activity, and

(2) an investigation of the products of the activity of the bacterial organisms found in conjunction with the worms.

Duration of activity in hydrogen.

A number of worms (*Ascaris lumbricoides*) freshly taken from the gut of a pig, were washed in warm Ringer solution, and placed in bottles containing a 1 % solution of sodium chloride boiled to expel air.

Two worms of equal size were placed in each bottle, and the bottles closed by means of a cork through which inlet and exit tubes were passed. In this and subsequent experiments great difficulty was encountered in keeping the inlet tubes clear, as, unless care was taken to prevent it, the worms climbed up the tube until in the end one became firmly fixed in it. This is an interesting example of what appears to be a definite purpose in the movements of these creatures. A slow stream of hydrogen was passed continually through the solutions, and the temperature maintained at 37°. The hydrogen after passing through the bottles was passed through a wash bottle containing baryta.

After the worms had become accustomed to their surroundings, they ceased to be active, and except for slight occasional movements remained so for 5–6 days, the only definite movements being those in connection with climbing the inlet tubes. At the end of this time the bottles were opened and allowed to stand in air, when within a short time the worms began to move freely, and this movement was increased if they were transferred to clean saline. The wash bottles containing baryta showed that carbon dioxide had been produced in considerable quantities. The sodium chloride solution in which the worms had lived had a characteristic unpleasant smell resembling that of the lower saturated fatty acids. The solution also gave qualitative tests for lactic acid.

These experiments confirmed in the first place Bunge's observations that the worms became dormant, and also in general the observations of Weinland.

It remained now to test whether if the worms were stimulated to continuous activity, they would live as well in hydrogen as in air. In order to do this two electrodes were introduced into the bottles and connected with the secondary circuit of an induction coil. By this means an interrupted current was passed through the solution from time to time, as often as was found necessary in order to keep the worms in movement. The circuit was arranged so that as nearly as possible equal stimulation was given in each containing vessel.

Three bottles were used each containing two worms. A continuous stream of hydrogen was passed through two of the bottles, whilst through the third a similar stream of air was drawn by means of a filter pump. The liquid used in the bottles was mammalian Ringer solution. All the experiments gave similar results, the following being a typical example of the findings:

Date 1924	Time	Remarks	Bottle 1 Worms in air	Bottle 2 Worms in hydrogen	Bottle 3 Worms in hydrogen
4. iii.	3 p.m.	Experiment started	All worms moving freely but slowly without stimulation		
	3.45	Continuous stimulation for 5 mins.	Violent movement	Responded to stimulus, but not so violently as worms in air	
	3.50–4.40	No further stimulus	Continued violent movement	Little further movement	
	4.40	Stimulation for 2 mins.	Very violent movement	Moving slowly	
	5.15	,,	,,	,,	
	5.45	,,	,,	,,	
	6.30	,,	,,	,,	
5. iii.	10 a.m.		All worms quite still		
	10.15	Stimulation for 5 mins.	Fairly active	Only a very slight movement	
	11.15 and every hour to 6 p.m.	Stimulation for 2 mins.	,,	Slightly more movement but very weak and jerky	
. iii.	10 a.m.		All worms quite still		
	10.30	Stimulation for 5 mins.	Considerable movement increasing with stimulation	No movement	A few slight tremors
	10.40	Air admitted to bottle 2			
	10.45		Moving easily	Slight movement	Quite still
	10.50	Stimulation for 2 mins.	,,	Slight movement confirmed	,,
	11.30	,,	,,	Movement increased	,,
	12 noon	,,	,,	,,	,,
	1 p.m.	,,	,,	,,	,,
	2	,,	,,	Movement less marked	,,
	3	,,	,,	Movement entirely stopped	,,
				It appears that the worms in both these bottles were dead	
iii.	10 a.m.	Stimulation for 2 mins.	Moving easily	No response whatever in either bottle	

Three other experiments were carried out, the result of one being identical with that given above, whilst in the other two where stimulation in hydrogen was not carried so far before the admission of air, the worms to which the air was admitted recovered completely. In no case did the worms which had a continuous supply of air cease to move, and in every case those which were entirely deprived of air became motionless in times varying from 24–48 hours.

From these experiments it appears reasonable to conclude that the worms are incapable of continuous movement in the absence of oxygen or its equivalent, and that it is only by avoiding movement that they are able to live for long periods in atmospheres of inert gases.

One criticism which might be made of these experiments is that the oxygen is necessary only for the excitation mechanism which is stimulated by the current, and not for the actual muscular movement. If this were the case, however, it would be expected that the worms would commence to move freely almost as soon as the oxygen was admitted, whereas they recover only very slowly.

It might also be suggested that the stimulating current by becoming partially rectified may have introduced small quantities of electrolytic oxygen which might have the effect of rendering possible movement in the anaerobic vessels longer than would otherwise be possible. This does not appear to be likely however as the stream of hydrogen would rapidly wash away any oxygen formed.

Examination of the products of bacterial action.

Several attempts were made to obtain the worms in a bacteria-free condition in order that they might be examined without the confusion consequent upon bacterial activity. All common antiseptics in concentration sufficient to render the worms free from bacteria had at the same time a lethal effect on them. Proflavine was tried in concentrations necessary for effective sterilisation (1 in 10,000), but the worms died in 1–2 days showing marked staining by the antiseptic. It was therefore decided to prepare a culture of the bacteria and to test this alone, as the worms could not be tested without the bacteria.

A peptone culture was prepared of the fluid in which the worms had lived. A number of mixed colonies resulted, and a solution containing salt, peptone and glucose was inoculated with bacteria from as many of these colonies as possible. After 2 days at 37° the solution was examined and found to smell unpleasantly. A part of the acid in the solution was distilled in steam and on examination appeared to be a mixture of saturated fatty acids (acetic, butyric, etc.) such as those which are characteristic of the solution in which the worms have lived.

The further examination of these acids was not attempted as it seemed that no useful purpose would be served by this investigation unless pure cultures were first obtained; it had been established that the products of the bacterial action were at least very similar to those of the worms plus bacteria.

Discussion and conclusions.

The general conclusion to be drawn from the experiments described appears to be, that although the worms are capable of prolonged existence in the absence of air, they achieve this only by cutting down their movements to a minimum, and that for their normal metabolism they require a supply of oxygen or its equivalent. Weinland's observations in themselves are confirmed, but he was misled by this faculty of "suppressed animation" into attributing to the worms an anaerobic mechanism. The metabolism of the worms is in general normal and the presence of the volatile fatty acids is due to bacterial activity.

This conclusion involves the assumption that the worms have the power of reducing their rate of metabolism to a very small value at 37°. Although reductions in metabolic rate are common in many animals at low temperatures during periods of hibernation, the present case appears to be without precedent. This may be due to the fact that the *Ascaris* has no circulatory system and is maintained at a constant temperature in the gut of its host.

It was hoped that calorimetric measurements of the metabolic rate in oxygen and in hydrogen could be made in order to confirm this point, but owing to the accompanying bacteria this was found to be impossible. It is proposed at a future date to carry out some measurements on the respiratory exchanges of thin strips of muscle tissue from the worms, which have been rendered bacteria-free, and thus to establish certainly whether oxygen is metabolised or not.

There are two further points which require comment. The first of these is the question of the oxygen supply to such an organism living in the gut of a mammalian host. It has been maintained that the oxygen pressure in the gut is almost negligible, and it has been generally assumed in consequence that there is no oxygen available for any organism living in the gut. A more careful consideration of the circumstances suggests however that this view may not be wholly correct. The gut is in intimate contact with the portal circulation, which during the period of digestion is highly oxygenated (the epithelial cells of the gut wall must themselves use oxygen), and hence it is to be expected that the gut and the neighbouring gut contents will have available an appreciable oxygen supply. The strongly reducing character of the gut contents is sufficient to explain the difficulty of finding oxygen in the intestinal gases. That molecules much larger than oxygen can be absorbed from the blood stream by parasites living in the gut is illustrated by an observation made by Dr Wollard (appended as a note to this paper), in which a tapeworm was found to have absorbed methylene blue, which had been injected intravenously into its host.

The other point requiring comment is the utilisation by the bacteria in the saline of carbohydrate derived from the bodies of the worms. The only conclusion which seems possible is that the carbohydrate (possibly as lactic acid) is excreted by the worms through their simple gut wall into the saline. The absorption of water from 1 % saline, and the extreme sensitivity of the worms to lower concentrations, suggests that the gut contents of the host rarely fall below that concentration under normal conditions. Hence if the gut wall of the worms is permeable in both directions, the conditions would be favourable to the excretion of the carbohydrates which are present in the body cavity of the worms in high concentration.

In conclusion the author wishes to acknowledge his thanks to Prof. H. S. Raper for the interest which he took in the work, the major part of which was carried out in his laboratory at Manchester.

NOTE BY H. WOLLARD.

ON THE ABSORPTION AND SUBSEQUENT REDUCTION BY A TAPEWORM OF METHYLENE BLUE FROM THE GUT OF ITS HOST.

A rat which had been injected for another purpose with 0·05 % methylene blue in normal saline at body temperature and immediately opened up, was found to contain a large tapeworm in its small intestine. The intestines had rapidly become blue. (Structures such as the nerve endings in muscle had been readily stained.) Portions of the tapeworm which was perfectly colourless were withdrawn and placed in air. These portions showed slight contractions and relaxations, and after they had been exposed to air for some time they exhibited a uniform blue colour of moderate density. They were not so deeply stained as the intestine, but the colour was approximately the same as that shown by the surface of the striated muscle. Several proglottides were placed on a glass slide and covered by a cover slip. The segments began immediately to decolorise and were soon quite colourless. This process of coloration and decoloration could be watched under the microscope by raising and lowering the coverslip, and it was observed to be taking place in the cytoplasm of the cells. The particles of the dye accumulated as perinuclear rings, and there underwent decoloration.

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XCI. THE USE OF THE GLASS ELECTRODE IN BIOCHEMISTRY.

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The purpose of this paper is to describe the glass electrode as a means of measuring hydrogen ion activity. By it a p_H determination can be made on 0·5 cc. or less of liquid, whether clear, coloured or turbid, easily reducible, or unstable in air, with an accuracy of 0·02 p_H, the experiment taking less than 5 minutes, and the sample being returnable chemically unchanged at the end.

Throughout this paper the term c_H is taken to designate the hydrogen ion activity, and p_H the negative logarithm of the hydrogen ion activity.

The possibility of the use of glass as an electrode for hydrogen ions was first discovered by Haber and Klemensiewicz [1909] and was confirmed by Freundlich and Rona [1920]; the glass electrode was compared with a hydrogen electrode by Hughes [1922], v. Steiger [1924] and Briske [1924] and correlated with the chemical composition of the glass by Horovitz [1923] and Schiller [1924] and described with some experimental detail by Brown [1924].

The chemical and physical theories as to the behaviour of the glass are not considered here, as the true explanation does not yet seem to have been found beyond dispute. Used empirically the method is simple, needing only extreme care in the technique. There has been some hesitancy in the adoption of the glass electrode—a hesitancy quite out of proportion to the unique advantages and facile manipulation it can present when once carefully set up.

General Principles.

Glass is used as a membrane to separate two solutions of different hydrogen ion activity. If the membrane be thin enough—about 25μ in its thinnest part—the electromotive force between the two solutions set up by the difference in activity may be measured. If the c_H of one solution be known, that of the other may be calculated from the electromotive force. Contact is made with the solutions by the arms of two saturated KCl-calomel electrodes arranged to oppose one another. The E.M.F. arising from this cell combination is balanced and measured potentiometrically.

The technical difficulties are caused by the high resistance of the glass membrane (50–100 megohms), the electric current naturally tending, if possible, to short-circuit the electrode by leaking through what would normally be considered an insulating medium, and especially across dirty or moist surfaces of insulators. Dirt is always a difficulty, but the trouble with moisture varies, being more obvious in summer than in winter, as, in the summer, room air has a greater relative humidity.

Fig. 1 shows the general arrangement of connections.

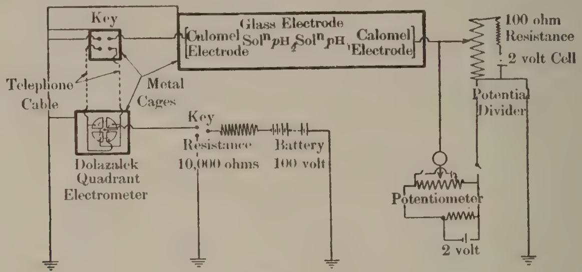

Fig. 1.

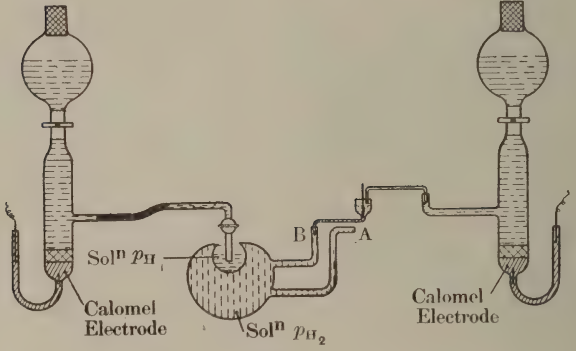

Fig. 2.

The Glass Electrode.

The electrode must be so designed as to present a very thin glass membrane, so held by stronger glass that it may separate two solutions. The simplest form used by all previous workers was that of a small bulb blown at the end of a piece of glass tubing. The one solution was put inside the bulb, while the other solution was held in a beaker in which the bulb was immersed. This shape has several disadvantages; for example, some 20–30 cc. at least of the liquid of unknown p_H are required in the beaker; it is not easy to fill and refill the bulb attached to the tubing as there is no exit for the air; and moreover the bulb is very easily broken. It is naturally under some strain, being of the limiting thickness capable of holding together as a bulb. In stirring the solution in the beaker, or in washing and drying the bulb, great danger of breakage is incurred.

A form of electrode found very convenient for biological purposes is illustrated in Fig. 2. The membrane in this case is in the shape of a deep spoon sucked inside a bulb of thicker glass. This is very easily glass-blown after a little practice. The solution of known p_H may be filled into the outer bulb by turning the electrode on its side and putting the tube *A* under the jet of a pipette or burette, the air in the bulb escaping through *B*. The other solution, that of unknown p_H for example, is put into the spoon. The advantages of this form are self-evident. Filling, washing and emptying the inside solution are simple operations. The membrane blown in this way seems to be under less strain than when blown at the end of a tube, and does not break so easily, and the quantity of liquid required for the spoon is very small. The total capacity of the spoon is about 1 cc. and it need never be more than half-full. A smaller quantity than 0·5 cc. may be used if necessary. If the liquid under experiment is liable to air oxidation or to loss of CO_2 it may be covered with liquid paraffin without affecting the E.M.F.

Another form of electrode found convenient, especially when the samples of unknown p_H solution can be allowed to flow successively through the electrode or when it is desirable to work in a thermostat, is shown in Fig. 3. The solution of known p_H is placed in the outer vessel and the unknown solution in the narrow tube going through the ground stopper. A thin bulb is sucked into an enlarged part of this tube. The total capacity of the tube is about 1 cc.

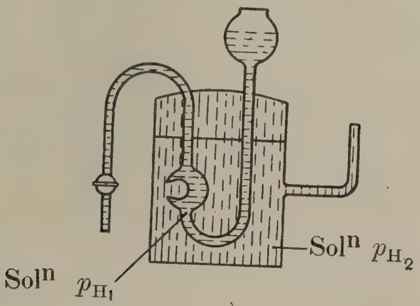

Fig. 3.

The glass used for the electrodes was an ordinary soft soda laboratory glass. Haber used originally a soft Thüringian glass. Horovitz [1923] and Brown [1924] found that certain borosilicate glasses act as electrodes for sodium as well as hydrogen in certain ranges of p_H, or as mixed electrodes. I have found that certain glasses which acted as mixed sodium and hydrogen electrodes, when a sodium phosphate buffer mixture was used as the known solution, acted accurately as $H^{\cdot}$ electrodes when the Na phosphate solution was replaced by a K phosphate or a KCl solution. No results could be obtained with "Durosil" glass, or with pure silica electrodes, presumably because the resistance of these membranes was too large even when thin.

The Calomel Electrodes.

The calomel electrodes were of the type shown in the diagram and were filled with saturated KCl solution four times re-crystallised. The calomel was prepared electrolytically according to instructions given by Clark [1923].

The contacts with the glass electrode solution were made in the manner shown, special care being taken to avoid diffusion of the heavy saturated KCl into the other solution, which may alter the E.M.F. by 2 or 3 millivolts. A tap, ungreased in the middle race and turned off, allows sufficient KCl to pass for electrical contact, but allows no flow. Another useful device to avoid diffusion is to leave a small bubble in the end of the glass capillary tube dipping into the electrode, the sides of the glass being wet enough to conduct. Convenient latitude in manipulation is obtained by attaching the arm of the calomel electrode to a rack and pinion, so that it can be readily lowered to, or raised from, the solution in the spoon of the glass electrode. The contact to the other calomel electrode is by means of narrow tubes and a small cup with a ground stopper. As a further precaution these tubes are removed from the electrode between readings.

The Potentiometer.

Theoretically only one potentiometer should be necessary for the measurement of the E.M.F., but in practice it is convenient to use a Pye rotary potential divider, together with a 2-volt accumulator and a 100 ohm resistance, to tap off the current required to balance that from the electrodes, and a potentiometer to measure accurately the current thus tapped off. The latter is standardised by means of a standard cell, and is used in conjunction with a small pointer galvanometer; or a combination apparatus, such as that supplied by the Cambridge Scientific Instrument Co. for hydrogen ion concentration measurement, may be employed.

The Electrometer.

A quadrant electrometer of the Dolazalek type has been used as null point indicator in the electrode circuit. The difficulties which may be encountered in adjusting the instrument to give reliable and sensitive readings are described in the paper by Brown [1924]. A Lindemann electrometer, which is small and portable, is unfortunately not sensitive enough for the purpose.

The Switches and Connections.

Owing to the high resistance of the glass electrode, and the sensitivity of the electrometer, special precautions have to be taken with regard to insulation and to shielding. The high tension battery used for charging the electrometer needle was supported on paraffin wax blocks, and was on a wooden table separated by an air gap from the table carrying the electrodes. The latter were enclosed in an earthed cage of wire netting. The glass electrode was supported on a block of wax; and, as in the case of the calomel electrodes, was insulated by an amberite rod held in an earthed metal stand. All connections to earth were of bare thick copper wire which was soldered where required. In the first instance, other connections were of varnished copper wire suspended by paraffined string, junctions being sunk in paraffin wax and the whole surrounded by a large cylinder of earthed wire netting. This arrangement was found unsatisfactory, as in time the wax became dirty, and in wet weather the surface was moist and the amount of leakage considerable. Such difficulties were entirely avoided by the use of lead shielded telephone cable. The Callendar Cable and Construction Co. kindly presented us with a length of their dry core cable, in which the insulating medium is virtually the air in the pores of paper dried in a vacuum at 250°. They also sent two wooden boxes soaked in wax, in which wax-embedded connections to lighter cable leading to the electrometer and to the switch could be made. This lighter cable for small length connections was made by putting varnished copper wire through narrow waxed glass tubing; this was surrounded with rubber tubing, and all the space inside filled up with wax. The rubber tubing was sheathed on the outside with copper tape, which could be earthed, as was the lead sheath of the heavier cable. Care was taken that the wax used had never been heated above its boiling point, when its insulating properties are impaired.

This arrangement eliminated all leakage troubles satisfactorily and allowed the work to be carried on even on wet days.

Porcelain switches embedded in wax were unsatisfactory for the reason given above, *i.e.* that surface leakage starts after a time on account of dirt and moisture. For charging the electrometer needle with 100 volts, and for discharging it, an ebonite switch with brass conductors and ivory handles was used. For connecting the glass electrode to one or other pair of quadrants of the electrometer, and for earthing these, a switch was made having amberite insulators. The terminals on the ebonite top of the switch were surrounded by amberite collars, and were covered with earthed metal shields.

Manipulation.

Newly-blown glass electrodes require careful cleaning with chromic acid, steaming for about 2 hours and soaking in distilled water for 24 hours before they can be used. Even after this treatment there may be a potential at the glass surface of 40 millivolts or more, which decreases slowly with time until after about 2 days it reaches a constant level of 4 or 5 millivolts which it will retain for weeks. (This contact potential may possibly be due to a state of strain in the glass.)

The system may be represented as follows:

Hg	Hg_2Cl_2 in	KCl	Sol^n	Sol^n	KCl	Hg_2Cl_2 in	Hg
	sat^d KCl	sat^d	p_{H_1}	p_{H_2}	sat^d	sat^d KCl	

Glass membrane

The E.M.F. of the combination is given by

$$E = \frac{RT}{F}(p_{H_1} - p_{H_2}) + Eg,$$

when E is the total E.M.F., Eg that occurring at the glass membrane. If E and Eg are measured in millivolts, $\frac{RT}{F}$ is equal to 57·7 at 18°.

One method of finding the value of Eg is to put the same solution on both sides of the glass so that $p_{H_1} = p_{H_2}$. Practically, however, the more accurate way of working is to have at hand a small quantity of a buffer solution of known p_H either freshly prepared from highly purified chemicals [Clark, 1923] or tested by the hydrogen electrode. The solution p_{H_2} may then be a stock buffer solution, say of potassium phosphates, whose p_H need never be accurately known. With this solution in the outer bulb and ½ cc. of the standard solution in the spoon, a reading of the E.M.F. (E standard) is taken. The standard solution is then emptied from the spoon, the latter washed several times with distilled water and filled with the solution of unknown p_H (p_{Hx}). The E.M.F. is again measured (E_x). p_{Hx} is then readily calculated from the equation

$$p_{Hx} = p_{H\text{std.}} \pm \frac{E_{\text{std.}} - E_x}{\frac{RT}{F}}$$

$$= p_{H\text{std.}} \pm \frac{E_{\text{std.}} - E_x}{57{\cdot}7} \text{ at } 18°.$$

This method of standardisation eliminates any error due to slight inequalities between the calomel electrodes or to liquid-liquid contact potentials. One standardisation at the beginning of each day's series of experiments is sufficient, but it may conveniently be repeated at the end of the day for confirmation.

In measuring the E.M.F. it is often quicker to balance only approximately with the potentiometer and then to read the remaining small deflection of the electrometer; the sensitivity of this instrument may be determined by measuring the change in deflection for a known change in E.M.F.

Lastly, the need for great cleanliness cannot be too strongly emphasised.

Application.

The technique described has been employed for some 450 estimations of the p_H of various substances. A few typical examples are:

Material	p_H
Blood (human-oxalated)	7·42
Blood (dog-defibrinated)	7·75
[Blood by Dale-Evans' method	7·73]
Flour (turbid mixture with H_2O) ...	6·15
Bean seedlings (aqueous extract) ...	5·87
Phosphate solution	10·5
Phosphate solution	7·37
[Phosphate by hydrogen electrode ...	7·39]
Sycamore leaves (aqueous extract) ...	4·88
[Sycamore by hydrogen electrode ...	4·91]
Culture medium (broth, etc.)	5·95

I am indebted to Dr Chibnall for the measurements indicated on the hydrogen electrode.

A comparison also of the "buffering powers" of heart and skeletal muscle, by means of titration curves, has been possible by the use of the glass electrode. These results will be published later.

I am deeply grateful to Prof. A. V. Hill, F.R.S., for much help and advice during the course of this research, which was carried out with the aid of grants from the Scientific and Industrial Research Department and the Medical Research Council.

SUMMARY.

1. The use of the glass electrode as an instrument for biochemical investigation is discussed, and its technique described in detail.

2. Two modified forms of electrode requiring only 0·5 cc. of liquid for examination are described.

3. Examples are given of the use of the electrode for various biochemical substances.

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XCII. THE "THIRD COMPONENT" OR HEAT-STABLE FACTOR OF COMPLEMENT.

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The separation of complement by various methods of protein precipitation into mid-piece and end-piece, or globulin and albumin fractions, has been well established.

Ferrata [1907] and Brand [1907] effected the separation by dialysis, Liefmann [1909] by saturation with CO_2, and Sachs and Altmann [1909] by the use of dilute hydrochloric acid. Although the two fractions may be present as a single component in the original serum, the method of separation by dialysis appears to support the view that they are separate entities from the first; other evidence is in favour of some loose chemical or physical union between them. On the other hand, Browning and Mackie [1913] contend that the so-called mid-piece and end-piece fractions do not represent constant entities, basing this view on inability to get perfect separation on every occasion by the CO_2 method of Liefmann and on the results given by their ammonium sulphate separation. It seems, however, too exacting to expect the simple methods of protein separation to give results comparable with those of inorganic methods, although our experience of these methods, especially Liefmann's, has been rather more satisfactory. We find in almost every experiment that the separate fractions give practically no complement action whatever even when quantities corresponding to four times the minimum haemolytic dose (M.H.D.) are used, whereas, when combined, they show practically the same activity as the original complement.

Von Dungern [1900] was the first to describe a phenomenon whereby complement is inactivated by treatment with yeast cells. His observation was confirmed by Ehrlich and Sachs [1902], and Coca [1914] further showed that the inactivation was due to the removal of a relatively heat-stable component and not to the addition of an inhibiting factor. He found that yeast-treated complement could be reactivated by the addition of normal guinea-pig serum which had been inactivated by heating at 56°, or of either the mid-piece or end-piece. He also concluded that a similar inactivation of complement by means of cobra venom, observed by Braun [1911], Omorokow

[1911] and Ritz [1912], was due, not to absorption of the heat-stable component as in the case of yeast, but to inhibition of the function of this factor. He observed that cobra-venom-inactivated serum could at times supply the heat-stable factor to yeast-inactivated serum. Browning and Mackie [1914] working with cobra-venom-inactivated serum found that the heat-stable or third component was present in the mid-piece but not in the end-piece. Jonas [1913] working with pigs' serum found that this serum was particularly rich in third component, which was present in both mid-piece and end-piece. Moore [1919], Coca [1920] and Hyde [1923] reported the discovery of a race of guinea-pigs whose complement was deficient in third component. The serum from these guinea-pigs showed practically no complement activity, but could be activated by the addition of small amounts of heated serum in the same manner as yeast-treated serum.

Although the theories of complement action assuming the presence of mid-piece and end-piece have gained general acceptance, the presence of the third component in complement seems to be doubted by many observers. Thus references are made to the "so-called" third component and at other times the facts relating to this factor are disregarded. Browning and Mackie [1913] consider that it is doubtful whether the assumption of a third component can explain all the phenomena and conclude that complement power is due to the interaction of many factors. Brooks [1920], on the other hand, considers that only one haemolytic substance is concerned, which is constantly being formed from some precursor (probably a lecithin), but he admits that this lytic substance is dependent for its action upon the state of the serum proteins. The importance of the protein constituents in serum is emphasised by the experiments of Jacoby and Schütze [1910] on inactivation by shaking, and by those of Michaelis and Skwirsky [1910, 2] and the present authors [1925] on the destruction of part of complement by proteolytic enzymes. There seems, indeed, sufficient evidence to warrant the view that the serum proteins play a much greater part than is suggested by Brooks.

The objects of the present work were:

(i) A fuller study of the action of yeast in removing third component.

(ii) An attempt to determine the actual nature of the process and to obtain a more satisfactory and constant agent than yeast.

(iii) To determine whether the third component is definitely associated, chemically or physically, with either of the two well-established protein fractions.

Dr R. R. Hyde of The Johns Hopkins University, Baltimore, kindly sent us some dried Fleischmann's yeast, which he recommended as an inactivating agent and this proved to be much more efficient than ordinary baker's yeast. Both yeasts, however, inactivated guinea-pig serum when used in excess, so that a paste was formed. The serum obtained by centrifuging the mixture after incubation for 2–3 hours was usually quite devoid of complement action and its activity could be restored by adding small amounts of guinea-pig serum inactivated by heating at 56°. The method was, however, by no means certain, since much depended on the amount of yeast used and the time of incubation. The only way to obtain an inactive serum with certainty was to prepare a large quantity of yeast-serum mixture and to withdraw samples after various periods of incubation. It was while endeavouring to determine whether the inactivation process was an enzyme action that a better method was discovered. Yeast heated in saline at 100° for half to three-quarters of an hour proved a better agent than fresh yeast and a preparation of zymin powder similarly heated was the best inactivator found. Heated zymin was almost invariably successful in removing the third component from serum by 2 hours' treatment.

Before assuming that the process is one of adsorption several alternative possibilities must be eliminated:

(*a*) One of the enzymes of the yeast may destroy the third component. As already mentioned, prolonged heating of yeast and zymin at 100° improved their action so that an enzyme action appears unlikely. The possibility that the enzyme or enzymes concerned are intra-cellular and, therefore, more stable towards heat must, however, not be overlooked. In zymin powder the yeast although dead still retains some of its cellular structure.

(*b*) Inhibiting substances from the yeast may pass into the serum. Coca showed that this did not happen and our experience confirms his results. Further evidence against this theory was obtained by testing the action of yeast-treated sera on diminishing amounts of complement, when no inhibition of complement action was obtained.

(*c*) The yeast may raise the salt content of the serum so much that haemolysis is inhibited by osmotic action. This has been disproved in two ways. Yeast-inactivated serum requires exactly the same amount of distilled water to render it haemolytic for unsensitised red cells as does the corresponding normal guinea-pig serum, showing that the two sera are isotonic. Also, dialysis of yeast-treated serum against normal saline does not in any way affect its properties, *i.e.* its complete lack of complement activity and its activation by solutions containing the third component.

(*d*) The yeast may cause an acidity in the serum sufficient to precipitate the mid-piece or globulin fraction. As will be shown later this is not the case, since yeast-inactivated serum still contains mid-piece.

It seems certain therefore that yeast inactivates guinea-pig serum by combining physically or chemically with a heat-stable component of the serum.

It was thought possible that some other mechanical adsorbent might be substituted for yeast or zymin with the advantage that a more uniform reagent would be available. With this end in view, kaolin and charcoal, as typical adsorbents, were tried in place of yeast. Hecht [1923] states that kaolin adsorbs the mid-piece of complement most readily, but in our experiments both charcoal and kaolin when used in excess appear to remove all constituents of the serum and to have no specific effect on the third component. Kaolin indeed seemed to remove the third component with least certainty, since several samples of serum treated with it, whilst quite inactive alone, contained some heat-stable factor and gave haemolysis when added to inactive yeast-treated serum. A curious fact noted with both kaolin and charcoal was that the serum after treatment was slightly alkaline in reaction whereas with yeast the reaction was distinctly acid.

In order to determine the relation of the third component to the other fractions of complement, serum was separated into globulin and albumin fractions or mid-piece and end-piece (hereafter known as *M* and *E*) by Liefmann's CO_2 method with various modifications described later. Two clear-cut fractions were almost always obtained which showed no complement action separately. Solutions *M* and *E* were tried for complement action in combination with inactive sera, produced by the action of various yeast and zymin preparations, and both solutions usually proved to be activating agents, proving the presence of the third component in each. It was found, however, that *E* had to be used, in nearly every case, in greater quantity to produce complete haemolysis and occasionally no third component could be demonstrated in this fraction. *M* and *E* were then heated at 56° until they contained no mid-piece or end-piece and practically the same result was obtained with the heated as with the unheated solutions.

It seems likely, therefore, that methods of protein separation have no specific action on the third component but that part of the latter is carried down with the globulin precipitate, the proportion varying with each individual serum and perhaps with the conditions of the experiment. In general, we have observed that in sera rich in heat-stable factor, both fractions contain this activating principle, and in cases where there is only a little, the globulin precipitate (*M*) appears to carry down the greater part, if not all.

Serum inactivated by yeast or zymin has been shown to contain both *M* and *E* but not the third relatively heat-stable component, and therefore separation of the inactive serum by CO_2 should give fractions free from the heat-stable factor. The separation was carried out and the mid-piece and end-piece fractions (M^1 and E^1) so obtained were found to have no complement action singly or when added together in varying proportions. E^1 could on every occasion be activated by small amounts of *M* from fresh serum, and M^1 was sometimes activated by *E*. The latter phenomenon only happened with sera which were shown otherwise to contain a relatively large amount of third component, part of which was present in the end-piece fraction. When no heat-stable factor was present in *E* no activation of M^1 by fresh end-piece was observed. These facts strongly support the theory that three factors are necessary for complement action, a globulin fraction (mid-piece), an albumin fraction (end-piece) and a third component or relatively heat-stable factor.

As a preliminary to further work on the third component we have attempted to activate yeast- and zymin-treated serum with aqueous and methyl alcoholic solutions of sodium oleate and egg-yolk lecithin and with solutions of egg white. Up to the present the results are entirely negative in every case, *i.e.* the inactive solutions could not thus be rendered specifically haemolytic for sensitised red cells.

EXPERIMENTAL.

The haemolytic system. The experimental work was duplicated in laboratories at Leeds and Newcastle. In the former, ox cells and rabbit anti-ox serum constituted the haemolytic system, and in the latter, sheep cells and horse anti-sheep serum. In all cases guinea-pig serum was used as the source of complement.

The minimum haemolytic dose of complement varied, but for the sake of convenience the experimental details will be given from one laboratory. The solutions to be tested were pipetted into small $3'' \times \frac{1}{2}''$ tubes, the bulk in each made the same (1 cc.) with 0·85 % saline, and 1 cc. of sensitised blood added. The results were read after 1 hour's incubation at 37° in an air incubator according to the following standards:

+ + + +	complete haemolysis	+ + +	intermediate degrees.
+ +	half-haemolysed	+	
–	no haemolysis.		

Preparation of zymin and inactivation process. Zymin powder was prepared by digesting baker's yeast with equal parts of alcohol and ether, filtering and repeating the process. Finally, the treated yeast was washed with alcohol and dry ether, dried at room temperature and passed through a fine mesh sieve. Thus a fine impalpable yellowish white powder was obtained. It is interesting to note that none of the preparations we made by this method contained any zymase as judged by its action on glucose.

Zymin was added to 10 cc. of 0·85 % saline in a centrifuge tube until a thin paste was formed. The mixture was heated in a boiling water-bath for $\frac{1}{2}$ to 1 hour and then centrifuged, the supernatant saline being poured off. Fresh guinea-pig serum was then added to the zymin deposit and the whole stirred with a glass rod to make a viscid emulsion. The tube was incubated at 37° and samples withdrawn at $1\frac{1}{2}$, 2, 3 and 4 hours. The serum was recovered from each sample by adding normal saline in an amount calculated to give 1/10 serum and centrifuging. Before using, the serum, which was definitely acid, was adjusted by means of a comparator to p_{H} 7·5. The amount of $N/10$ NaOH required to neutralise a volume equivalent to 5 cc. of the original serum was in the neighbourhood of 1·0 cc. Inactivation by unheated zymin and by yeast and heated yeast was carried out in a similar way. A curious fact occasionally noticed was that treatment with a greater quantity of yeast for the same time or for a longer period failed to inactivate the serum. No explanation of this phenomenon has yet been found.

The following table is typical of the results which have been repeatedly obtained:

Table I.

0·5 cc. 1/10 complement	+ + + +
0·5 cc. H_{20} (1/10 complement heated 20 minutes at 56°)	–
0·5 cc. H_{40} (1/10 complement heated 40 minutes at 56°)	–
0·5 cc. Z_1 (1/10 zymin-inactivated complement. 2 hrs.)	–
0·5 cc. Z_2 (1/10 zymin-inactivated complement. 3 hrs.)	–
0·5 cc. Z_1 + 0·5 cc. H_{20}	+ + + +
0·5 cc. Z_2 + 0·5 cc. H_{20}	+ + + +
0·5 cc. Z_1 + 0·5 cc. H_{40}	+ + + +
0·5 cc. Z_2 + 0·5 cc. H_{40}	+ + + +

Similar results were obtained with zymin and yeast which had previously been heated in the dry state at 100° in an air oven for 1 hour and which were then washed with normal saline.

Preparation of mid-piece and end-piece and their effect upon complement inactivated by zymin or yeast. Complement was diluted 10 times with distilled water, cooled in ice and saturated with CO_2 by passing the gas for 10–15 minutes. The globulin separated as a flocculent precipitate and was removed by centrifuging. The supernatant fluid containing end-piece (*E*) was decanted into a flask, sufficient 10 % saline added to render it isotonic with normal saline, and then the flask was evacuated twice to remove CO_2. The mid-piece (*M*) precipitate was lightly washed twice with distilled water, and then dissolved in a volume of normal saline equal to that of the end-piece. Both solutions were finally adjusted in a comparator to p_H 7·5. *E* was always quite acid (about p_H 6); *M*, although slightly acid, was unbuffered. The importance of removing the CO_2 and adjusting these solutions seems to have been overlooked by many workers on complement. Excess of CO_2 has a marked anti-complementary action and although in most of our solutions the acidity was not sufficient to affect the results, it is obviously desirable that all solutions should approximate in reaction to normal serum. Evacuation of *E* removes most of the CO_2 and for ordinary purposes this treatment is perhaps all that is necessary.

Table II.

$\left.\begin{matrix} M_{15} \text{ and } M_{30} \\ E_{15} \text{ and } E_{30} \end{matrix}\right\} = E$ and M heated at 56° for 15 and 30 minutes.

0·2 cc. 1/10 complement	+ + + +	0·5 cc. *E*	–
0·5 cc. 1/10 complement	+ + + +	1·0 cc. *E*	–
0·5 cc. *M*	–	0·5 cc. *M* + 0·5 cc. *E*	+ + + +
1·0 cc. *M*	–	0·2 cc. *M* + 0·2 cc. *E*	+ + + +
0·5 cc. Z_1 + 0·5 cc. H_{20}	+ + + +	0·5 cc. Z_1	–
0·5 cc. Z_1 + 0·5 cc. H_{40}	+ + + +	0·5 cc. Z_1 + 0·5 cc. *M*	+ + + +
0·5 cc. H_{20} + 0·5 cc. *E*	–	0·5 cc. Z_1 + 1·0 cc. *M*	+ + + +
0·5 cc. H_{20} + 1·0 cc. *E*	+	0·5 cc. Z_1 + 0·5 cc. *E*	+
0·5 cc. H_{20} + 0·5 cc. *M*	–	0·5 cc. Z_1 + 1·0 cc. *E*	+ + +
0·5 cc. H_{20} + 1·0 cc. *M*	–	0·5 cc. Z_1 + 0·5 cc. M_{15}	+ + + +
0·5 cc. H_{40} + 0·5 cc. *E*	–	0·5 cc. Z_1 + 1·0 cc. M_{15}	+ + + +
0·5 cc. H_{40} + 1·0 cc. *E*	–	0·5 cc. Z_1 + 0·5 cc. E_{15}	–
0·5 cc. H_{40} + 0·5 cc. *M*	–	0·5 cc. Z_1 + 1·0 cc. E_{15}	+ +
0·5 cc. H_{40} + 1·0 cc. *M*	–	0·5 cc. Z_1 + 0·5 cc. M_{30}	+ + + +
		0·5 cc. Z_1 + 1·0 cc. M_{30}	+ + + +
		0·5 cc. Z_1 + 0·5 cc. E_{30}	–
		0·5 cc. Z_1 + 1·0 cc. E_{30}	+

Effect of excess of CO_2 *on complement action.* Michaelis and Skwirsky [1910, 1] have shown that the action of complement varies with the reaction of the solution. By the aid of phosphate buffer mixtures these authors found that an acid reaction inhibits complement action and that the reaction proceeds best in neutral or faintly alkaline solutions. In view of this we have investigated the effect of CO_2 on the action of decreasing amounts of guinea-pig complement. Normal saline was saturated with CO_2 and this solution added to varying strengths of diluted normal guinea-pig serum.

Table III.

0·5 cc. 1/10 normal complement		+ + + +
0·5 cc. ,, ,,	+0·5 cc. CO_2-saturated saline	+ + + +
0·5 cc. ,, ,,	+1·0 cc. ,, ,,	+ + + +
0·5 cc. 1/20 normal complement		+ + + +
0·5 cc. ,, ,,	+0·5 cc. ,, ,,	+ + + +
0·5 cc. ,, ,,	+1·0 cc. ,, ,,	+ +
0·5 cc. 1/30 normal complement		+ + + +
0·5 cc. ,, ,,	+0·5 cc. ,, ,,	+ +
0·5 cc. ,, ,,	+1·0 cc. ,, ,,	–
0·5 cc. 1/40 normal complement		+ + + +
0·5 cc. ,, ,,	+0·5 cc. ,, ,,	–
0·5 cc. ,, ,,	+1·0 cc. ,, ,,	–

Separation of zymin-inactivated serum into mid-piece and end-piece fractions. Zymin-inactivated guinea-pig serum was centrifuged, the supernatant liquid diluted with nine times its volume of distilled water and a slow stream of CO_2 passed through for 10–15 minutes. The two fractions were separated and dealt with as described for normal serum, the solutions being adjusted to p_H 7·5.

Table IV.

M and E = normal mid-piece and end-piece fractions.
M^1 and E^1 = mid- and end-piece from zymin-inactivated serum (by CO_2 separation).
M_{15}, M_{30}, E_{15} and E_{30} as in Table II.

M^1 and E^1 separately inactive in quantities of 0·5 cc. and 1·0 cc.

0·5 cc. M^1 + 0·5 cc. E^1	–
0·5 cc. M^1 + 0·5 cc. E	+
0·5 cc. M^1 + 0·5 cc. E_{15}	–
0·5 cc. E^1 + 0·5 cc. M	+ + + +
0·5 cc. E^1 + 0·5 cc. M_{15}	–
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. normal saline	–
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. M	+ + + +
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. M_{15}	+ + + +
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. M_{30}	+ + + +
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. E	+
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. E_{15}	–
0·5 cc. M^1 + 0·5 cc. E^1 + 0·5 cc. E_{30}	–

These results were obtained with a serum which did not contain a large amount of heat-stable factor and little was to be found in the normal end-piece fraction. Thus the zymin-inactivated serum contains the globulin and albumin fractions but no third component.

SUMMARY.

(1) The inactivation of guinea-pig serum by the action of yeast can also be effected by a zymin preparation. The serum so obtained can have its complement activity renewed by the addition of serum inactivated by heating at 56°.

(2) The possibility of explanations other than that already offered—viz. the removal of a "third component" or relatively heat-stable factor—has been investigated and disproved.

(3) Yeast and zymin heated in normal saline or in the dry condition at 100° for ½ to 1 hour are more efficient inactivators than the unheated substances, and thus the inactivation process does not appear to be one of enzyme action.

(4) Kaolin and charcoal are not similar to yeast in their action on serum.

(5) Zymin, like yeast, removes a heat-stable component of complement which is usually present in both mid-piece and end-piece as obtained by Liefmann's CO_2 method. The greater bulk of the heat-stable component is usually present, however, in the mid-piece and in some sera none can be found in the end-piece. No definite chemical or physical relationship appears to exist between this third component and the mid-piece and the end-piece.

Two of the authors (J. G. and A. W.) are indebted to the Medical Research Council for a grant in aid and we wish to express our thanks to Professors H. J. Hutchens and J. W. McLeod for facilities granted.

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XCIII. THE ESTIMATION OF ACETOACETIC ACID AND β-HYDROXYBUTYRIC ACID IN URINE.

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The principal methods for estimating acetoacetic acid, acetone and β-hydroxybutyric acid in body fluids are those of Shaffer-Marriott [1914] and of Van Slyke [1917].

The latter is a gravimetric estimation depending on the formation of a complex mercury-acetone compound, $3HgSO_4 . 5HgO . 2(CH_3)_2CO$. The former is an iodometric method based on the Messinger-Huppert titration.

In both methods the acetoacetic acid is decomposed with sulphuric acid, thus

$$CH_3.CO.CH_2.COOH \longrightarrow CH_3.CO.CH_3 + CO_2$$

and the β-hydroxybutyric acid is oxidised by a mixture of $K_2Cr_2O_7$ and H_2SO_4, thus

$$\begin{aligned} CH_3.CH(OH).CH_2.COOH &\longrightarrow CH_3.CO.CH_2.COOH + H_2O \\ &\longrightarrow CH_3.CO.CH_3 + CO_2 + H_2O \end{aligned}$$

In the Shaffer-Marriott method the acetone is made to react with hypoiodite to form iodoform; in the Van Slyke method it combines with mercuric sulphate to form the complex mercury-acetone compound.

For the estimation in urine the precipitation of pigments and sugar and any other aldehyde-forming substances is a necessary preliminary. Shaffer-Marriott used the lead acetate-ammonia method; Van Slyke used the copper sulphate-calcium hydroxide method.

This preliminary precipitation in urine is of the greatest importance in the estimation of β-hydroxybutyric acid, but is not absolutely essential in that of acetoacetic acid and acetone by the Shaffer-Marriott method. This statement is based on a large number of comparative estimations. In the Van Slyke method precipitation must always be carried out.

Hence in purely clinical work, using the former method, where only the acetoacetic acid and acetone are estimated, immediate distillation in acid solution with subsequent iodometric titration is all that is necessary. The yield in both methods for acetoacetic acid and acetone is generally agreed upon to be between 90 and 95 %.

In the estimation of β-hydroxybutyric acid, however, in which oxidising agents are used, preliminary precipitation can never be dispensed with, as the interfering substances present are fatal to accurate results. Even after precipitation it is by no means certain that what we are estimating is only this hydroxy-acid, although it is certainly the main constituent in, for example, urines of advanced diabetics.

In order to remove aldehydes from the distillate obtained from the oxidation, Shaffer-Marriott re-distilled with alkaline hydrogen peroxide before titration, a method which gives very constant yields of about 85 %. This percentage is not attained when dealing with quantities in the region of 5 mg. %. In the Van Slyke method the β-hydroxybutyric acid is obtained by difference, dichromate being used with one sample and not with a second, the difference in mass of the two precipitates representing the hydroxy-acid.

The principal objection to both these methods, particularly if serial determinations are to be made, is the long time necessary for a single analysis.

The Shaffer-Marriott method cannot be done under 3 or 4 hours and Van Slyke's method cannot be finished under 2½ to 3 hours. The high resistance of β-hydroxybutyric acid to oxidation is the main cause of these protracted periods.

In Van Slyke's method the filtration through the Gooch crucible is somewhat tedious and the transference of small particles of precipitate is always troublesome. In spite of this, however, we consider the Van Slyke a much more tractable method than the Shaffer-Marriott.

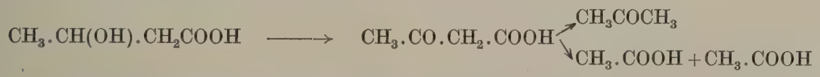
A further point about these methods is the relatively large volumes of urine used. In the Shaffer-Marriott an average of 50 cc. is taken and in the Van Slyke it is best to use 25 cc.

If, as in the work for which these methods were studied, only small volumes are available on which several analyses have to be made, such methods are not satisfactory.

Lastly, we may refer to the considerable expenditure of chemicals incident to these methods.

Engfeldt, using a micro-method and very small quantities of β-hydroxybutyric acid, has obtained yields of 70 % from urine and blood. Lublin [1922], combining the methods of Shaffer-Marriott and Engfeldt, used fractional volumes of urine and blood and claimed a fair accuracy for his micro-method.

The low yield in hydroxy-acid is due to the fact that the oxidation involves not only a production of acetoacetic acid and acetone but also a decomposition of acetoacetic acid with the production of acetic acid, in accordance with the well-known acid hydrolysis of ketonic acids. Thus:



$$CH_3.CH(OH).CH_2COOH \longrightarrow CH_3.CO.CH_2.COOH \begin{cases} \nearrow CH_3COCH_3 \\ \searrow CH_3.COOH + CH_3.COOH \end{cases}$$

Van Slyke has shown that, when $K_2Cr_2O_7$ and H_2SO_4 act upon β-hydroxybutyric acid at room temperature for some time, practically only acetic acid is produced; but at boiling temperature acetone is produced in considerable quantities.

In Lublin's method no preliminary precipitation of pigments is carried out in the analysis of urine, but the Folin-Wu tungstic acid precipitation is applied to the estimation in blood. For β-hydroxybutyric acid he assumes Engfeldt's yield of 70 %. He states that precipitation is unnecessary for ordinary purposes. This is, in our experience of this method, true for the estimation of the acetoacetic acid and acetone, but certainly not so for that of β-hydroxybutyric acid.

The volumes used by Lublin were 0·5 cc. urine and 0·2 cc. blood. In cases of severe acetonuria there is certainly no difficulty with 0·5 cc. urine, but 0·2 cc. blood has, in our hands, not yielded results which could be relied upon except in cases of severe diabetic ketosis.

Lublin distils directly into alkaline hypoiodite. Using such small volumes of material it would be futile to attempt re-distillation with alkaline H_2O_2 as in the Shaffer-Marriott method and so it is dispensed with. With regard to time of distillation Lublin gives 10 minutes for each stage, *i.e.* 10 minutes' distillation with acetic acid for the acetoacetic-acetone fraction and then a further 10 minutes' oxidation and distillation with $K_2Cr_2O_7$ and H_2SO_4.

Our experience of this method has shown that perceptible differences from the blank of the apparatus and reagents can be detected up to as long as 30 minutes from the commencement of each distillation, provided that rather larger volumes of distilled water are used in a larger distilling flask. Twenty minutes has been found the minimum time for a maximum yield.

In the oxidation for the β-hydroxybutyric acid, Engfeldt obtained his yield of 70 % using 1 g. of $K_2Cr_2O_7$ in 2 % solution at constant volume of urine.

Lublin used 20 cc. of 2 % $K_2Cr_2O_7$ in 20 % H_2SO_4.

The strength of dichromate used is of the first importance and we have found, using various solutions, that that used by Lublin is satisfactory. Of course the yields become less as we decrease the concentration of the β-hydroxybutyric acid, but for a given solution fairly constant results are obtainable.

Variations in time of oxidation and in strength of flame give rise to considerable variations in yield.

The method which we used in certain investigations to be published later was a modification of that proposed by Lublin.

The apparatus is a simple one and consists of a 250 cc. Kjeldahl flask, provided with an inlet tube made by fusing a long piece of thin glass tubing to a drawn-out test tube; the glass tubing is drawn to capillary bore and cut so as to reach almost to the bottom of the flask. Through this an air current can be drawn through the contents of the flask, and the dichromate-sulphuric acid mixture can also be introduced.

The flask is connected by a long delivery tube to a condenser which is connected to a 250 cc. Erlenmeyer flask, fitted with a glass tube with a perforated bulb and with a dilated tube, the latter being connected to a suction pump by a rubber tube. The rubber tube carries a screw clip for regulation of the air current. The Bunsen flame used for heating the flask is surrounded by a guard and can be adjusted by means of a screw clip.

Solutions required.

1. Basic lead acetate solution prepared according to Shaffer's directions; 180 g. lead acetate are dissolved in about 500 cc. distilled water and boiled; 110 g. of litharge are then gradually added until a clear solution is obtained with no remnants of the oxide. This is made up to a litre with distilled water. This solution on standing deposits some of the basic salt but the supernatant fluid is clear. The pipette used for this solution should be dry and should be cleaned immediately after use.
2. Strong solution of ammonium hydroxide (Sp. 0·880).
3. Solution of 25 % H_2SO_4.
4. Solution containing 2 g. $K_2Cr_2O_7$, 20 cc. concentrated H_2SO_4, made up with distilled water to 100 cc.
5. $N/50$ iodine
6. $N/200$ $Na_2S_2O_3$ freshly prepared from $N/10$ thiosulphate.
7. 25 % solution of nitrite-free NaOH.
8. 20 % HCl containing no free chlorine.

Into a small beaker 4 cc. urine[1], 4 cc. distilled water and 4 cc. of basic lead acetate solution are put and after a little agitation 2 cc. of ammonia are run in from a burette. Sugar, pigments, etc. are thus precipitated. In the case of urines containing little sugar less acetate is added and more distilled water, but the final volume is always 14 cc. After standing for 5 minutes the mixture is filtered into a test tube when a clear, colourless filtrate is obtained. 40 cc. distilled water are placed in the Kjeldahl flask and 3·5 cc. filtrate are run in from an accurately graduated pipette. This volume of filtrate is equivalent to 1 cc. urine. The volume of filtrate obtained depends on the amount of sugars, etc. to be precipitated. If this is inconsiderable as much as 8 cc. filtrate are obtained, but with urines of high sugar content a bulky precipitate is produced and perhaps as little as 4 or 5 cc. are obtained. In general, no difficulty is found in obtaining 3·5 cc. for the estimation.

5 cc. of 25 % H_2SO_4 are now added. If an excess of lead acetate is present a white precipitate of $PbSO_4$ is thrown down, but with a little practice this can be avoided by adjusting the volume of lead acetate added.

In the Erlenmeyer flask are placed 40 cc. distilled water, 10 cc. $N/50$ iodine and 2 cc. 25 % NaOH.

A slow current of air is drawn through the apparatus and the Kjeldahl flask is heated with the guarded Bunsen and the strength of flame adjusted so that at the end of 30 minutes about 15–20 cc. are left in the flask. This is merely a matter of a few trials. In this way the acetoacetic acid is broken down and the acetoacetic-acetone fraction distilled over with the formation of iodoform in the usual way. After 25 minutes' aeration and distillation, the current of air is increased and the flame turned off. After 30 minutes the receiver is disconnected, the delivery tube thoroughly washed down with distilled water, 5 cc. 20 % HCl are added and the contents titrated with freshly prepared $N/200$ thiosulphate using 1 % starch.

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[1] The only reason for taking so small a volume is that in the investigations for which this method was employed serial collections of urine at short intervals were made and, in general, not more than 7 or 8 cc. urine were available; in this volume several observations had to be made.

(It need hardly be emphasised that the blank of the apparatus and reagents must be accurately determined by repeated distillation. We have found an average blank of 1·5 cc. $N/200$ thiosulphate after 30 minutes' distillation.)

The volume in the Kjeldahl flask is now made up again to 40 cc. and another receiver containing the same quantities of iodine, etc. is connected. The flame is lowered a little by means of the screw clip and the $K_2Cr_2O_7$-H_2SO_4 mixture run in from the dropping funnel at a rate of about 1 drop every 2 or 3 seconds. Distillation and aeration are continued for 30 minutes when the receiver is disconnected and titrated as before. 1 cc. $N/50$ iodine = 0·193 mg. acetone = 0·50 mg. β-hydroxybutyric acid (70 % yield).

Notes on the Method.

1. The necessity of preliminary precipitation with the basic lead acetate solution, etc., at least in the estimation of β-hydroxybutyric acid, was shown by the following estimations in which the ketonic substances were determined with and without treatment with the lead. The figures given are volumes of thiosulphate required after distillation for 30 minutes into 10 cc. $N/50$ iodine, etc., as described above. I represents first distillation, II the oxidation of the hydroxy-acid.

10 cc. $N/50$ iodine required 39·6 cc. thiosulphate solution. Average blank for apparatus etc. 38·3 cc. thiosulphate solution.

	With precipitation		Without precipitation	
Urine	I	II	I	II
1	31·25	33·5	31·0	29·0
2	31·2	33·48	31·0	29·0
3	30·8	33·4	30·5	29·4
4	27·4	30·2	27·6	24·85
5	27·9	31·5	27·5	27·6

It is seen that the figures obtained by the two methods under I are very close to one another but this is not the case with those under II; that is to say, that without precipitation higher yields in "β-hydroxybutyric acid" will be obtained. The differences are as much as 4 or 5 cc. of thiosulphate which, assuming a yield of 70 %, would correspond to 0·5–0·6 mg. of β-hydroxybutyric acid.

This seems to us to be sufficient evidence of the necessity of preliminary precipitation.

2. Comparing the above method with Van Slyke's method we obtained the following results using diabetic urines. Acetoacetic acid and acetone only were estimated.

	Percentage acetoacetic acid and acetone	
Urine	Van Slyke mg.	Above method mg.
1	56·8	52·0
2	12·4	12·6
3	39·6	43·7
4	50·8	55·7

The general agreement is fair considering the widely different procedures.

3. The following experiments were carried out to find the yield of β-hydroxybutyric acid using the above method. For this purpose standard solutions of CaZn hydroxybutyrate were employed.

The CaZn salt was obtained as follows:

10 g. β-hydroxybutyric acid (Kahlbaum) was dissolved in 200 cc. distilled water and divided into two equal portions. To one portion excess of pure $CaCO_3$ was added and the mixture boiled for 30 minutes and then filtered. A clear brownish filtrate, consisting of calcium β-hydroxybutyrate, was obtained.

To the second portion of acid excess of freshly prepared well washed $Zn(OH)_2$ was added and the mixture boiled and filtered.

The two filtrates thus obtained were mixed and concentrated by gentle heat. The mixture was then poured slowly into excess of absolute alcohol, stirring vigorously. A finely divided white precipitate rapidly appeared. The alcoholic mixture was allowed to stand overnight and then centrifuged. The supernatant fluid was poured off, the remaining salt dissolved in a minimum of boiling water and again concentrated. Precipitation was carried out as before and the process repeated three times.

The pure white double salt thus obtained was placed in a desiccator and finally dried, by gentle heat, in a hot air oven to constant weight.

Analysis of the salt was carried out by incineration, the formula

$$(CH_3.CH(OH).CH_2.COO)_4CaZn$$

being assumed, and it was found to be pure.

517·696 g. of the double salt are equivalent to 416·288 g. of β-hydroxybutyric acid.

A solution containing 0·1969 g. of the salt in 200 cc. distilled water was prepared; 1 cc. therefore containing 0·9845 mg. of the salt and being equivalent to 0·7915 mg. β-hydroxybutyric acid.

Yield of β-hydroxybutyric acid from urine using the method described.

In each estimation a different urine was used and a control carried out with the urine alone.

10 cc. urine and 10 cc. of above solution of CaZn hydroxybutyrate were mixed. 2 cc. of this mixture, after allowing for the content of the urine itself, contained the equivalent of 0·7915 mg. β-acid.

10 cc. of the mixture and 10 cc. distilled water were treated with 10 cc. basic lead acetate solution and 5 cc. ammonia, filtered, and 7 cc. of filtrate used for the estimation.

Blanks for the urine alone were obtained in each case by using 5 cc. urine, 15 cc. distilled water, 10 cc. basic lead acetate and 5 cc. ammonia and estimating in 7 cc. filtrate.

In this way the following yields were obtained:

Urine	Theoretical mg.	Amount found mg.
1	0·7915	0·50
2		0·49
3		0·58
4		0·59
5		0·52
6		0·57
7		0·54
8		0·53
9		0·49
10		0·51
11		0·53

The average recovery was thus 0·53 mg., *i.e.* 67·2 %.

Similar experiments were carried out using volumes to give theoretical yields of 1·583 mg. β-hydroxybutyric acid, with the following results:

Urine	Theoretical mg.	Amount found mg.
1	1·583	1·14
2		1·13
3		1·11
4		1·18
5		1·18
6		1·18
7		1·18

The average recovery was thus 1·16, *i.e.* 73·3 %.

A fresh solution of CaZn β-hydroxybutyrate was now prepared containing 1·7527 mg. β-hydroxybutyric acid (2·18 mg. salt) per cc. and estimations carried out as before:

Urine	Theoretical mg.	Amount found mg.
1	1·7527	1·270
2		1·23
3		1·29
4		1·30

The average recovery was thus 1·27, *i.e.* 72·5 %.

SUMMARY.

1. A method of estimating the ketonic substances in small volumes of urine is described, which yields results comparable to those given by other methods using larger volumes.

2. The estimation of β-hydroxybutric acid by this method was investigated with the result that for quantities less than 1 mg. per cc. the yield was from 65–68 %, and for quantities between 1 and 2 mg. per cc. it was between 70 and 75 %.

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XCIV. A NOTE ON THE BLOOD-SUGAR LEVELS OF RATS FED WITH COMPLETE DIETS AND DIETS DEFICIENT IN VITAMIN B

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(*Received June 24th, 1925.*)

Introductory.

Based on the observation that the addition of carbohydrate to a beri-beri-producing diet fed to pigeons hastened the appearance of this disease, Funk [1914] suggested that the anti-beri-beri vitamin plays some part in carbohydrate metabolism. In the same year Funk and v. Schönborn [1914] described a hyperglycaemia and a shortage of glycogen in the livers in pigeons suffering from beri-beri.

In 1919 McCarrison stated that the absence of "anti-neuritic" food factors from the diet leads to an increase in the weight and adrenaline load of the adrenal gland, and to a state of acidosis due to the imperfect metabolism of carbohydrates and the acid fermentation of starches [1919]. On the basis of McCarrison's results, Funk [1920] pointed out that increased adrenaline in the circulation was probably responsible for the hyperglycaemia which he had obtained in another series of beri-beri pigeons (bled out under A.C.E. mixture). Funk's figures show in this experiment a higher glycogen content in the "beri-beri" livers than in the "normal control" livers—a result opposed to that which he and v. Schönborn had previously found. (See also the work of Tscherkes [1922] in this connection.)

From a study of the respiratory quotient of rats fed with diets deficient in vitamin B, Mattill [1923] concluded that there was no interference with the process of glucose combustion, traceable to this deficiency.

Since most of these observations were made on animals whose diets were unbalanced in other respects besides the lack of vitamin B, we thought it advisable to re-investigate the question of carbohydrate metabolism in rats fed on a well-balanced diet, deficient only in vitamin B. Our observations come under the following categories:

I. Blood-sugar levels in rats fed with complete diets and with diets deficient in vitamin B.

- (*a*) 1–3 hours after feeding (*i.e.* the "resting" level).
- (*b*) 24 hours after feeding (basal level).
- (*c*) After a glucose meal.
- (*d*) After a starch meal.

II. Glycogen content of the livers of rats fed with complete and with vitamin B-deficient diets, for different periods.

(*a*) 1–3 hours after feeding.

(*b*) 24 hours after feeding.

III. Food intake of rats fed with the diets used throughout this work.

Experimental.

Adult rats of 100–150 g. body weight were employed. The rats were kept several weeks in the laboratory before being used in the experiments, and those showing abnormal behaviour were discarded. The diet of the normal control animals was as under:

Rice starch ...	100 g.	Lemon juice ...	10 g.
Caseinogen ...	40	Salt mixture ...	10
Butter	20	Water	150
Yeast extract ...	10		

In the vitamin B-deficient diet the yeast extract was omitted and the caseinogen was purified by extraction with alcohol (boiling). In a later experiment meat extract (free from vitamin B) was added to this diet in an endeavour to match the flavour of the control diet. The vitamin B-deficient diet fed to an animal of 100 g. body weight produced coma in about 50 days [cf. Drummond, 1918].

Glycogen was estimated by the method of Pflüger, the livers being dropped into boiling alcoholic potash immediately after the death of the animal. For the estimation of blood-sugar the method of MacLean [Gardner and MacLean, 1914] was employed, necessitating 0·2 cc. of blood for an estimation. The samples were obtained from the brachial artery after stunning the animal, the use of anaesthetics being avoided. One sample of blood only could be taken from an animal in this way. Hence for the construction of the curves given in this paper 5–10 rats were used to obtain each point in a curve.

Blood-sugar levels.

The blood-sugar levels in rats fed with the vitamin B-deficient diet for different lengths of time are shown in Fig. 1. As is seen, the results indicated nothing abnormal in the deficient animals until the stage of the final collapse, when the blood-sugar fell to a starvation level. Contrary to Funk's experience with pigeons, there is no evidence of hyperglycaemia.

For the purpose of the feeding experiments contemplated, it was necessary to establish the basal level of the blood-sugar of the rat. It was found that a 24-hour fast brought down the level to a very steady basal level of 0·09 %. The simultaneous administration of adrenaline produced no lower results (Table I).

Table I. *Blood-sugar levels after a 24-hour fast.*

	Individual values (% glucose)	Mean
Normal rats	0·08, 0·09, 0·08, 0·10, 0·09, 0·10, 0·09, 0·09	0·09
Normal rats with simultaneous injection of 0·1 mg. adrenaline (intraperitoneal)	0·08, 0·09, 0·09	0·087
Rats 40 days on deficient diet	0·12, 0·13, 0·095, 0·13, 0·11, 0·11, 0·11	0·115

The blood-sugar values for the vitamin B-deficient rats are seen to be higher and more erratic than the corresponding figures for the normal rats. This may be due to absorption from the atonic intestine which, as one of us has shown [Gross, 1924] is subject to stasis in this condition. The livers of all these rats were empty of glycogen.

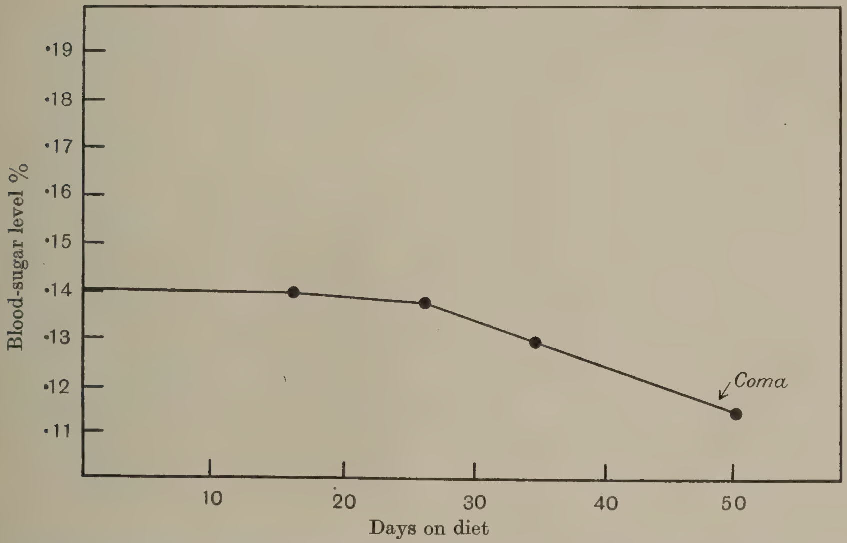

Fig. 1. The effect of vitamin B-deficient diet on the blood-sugar level of the rat.
NOTE.—The final value coincides with the basal level (*i.e.* level after 24 hours' fast).

Having established the basal blood-sugar of the rat when fed on normal diet and a diet deficient in vitamin B, the effect of a glucose meal was observed. The animals, after a 24-hour fast, were given a meal of glucose (with the aid of a stomach tube) the dosage being regulated to 500 mg. per 100 g. body weight. The animals were killed after periods of 15, 30, 45 and 60 minutes, and their blood-sugar estimated. The vitamin B-deficient rats had been 36–40 days on the deficient diet. The results are shown in Fig. 2.

When allowance is made for the lower basal level of the normal rat there is no significant difference between the two curves. Attempts to compare the rates of absorption of starch by the same method failed owing to the flatness of both curves.

A definite effect of vitamin B deficiency was found in the glycogen content of the livers of the animals (estimated by Pflüger's method). After a 24-hour fast the livers were empty of glycogen in all cases, but the values for rats taken straight from the cage showed a continued decline as a result of the deficient diet (Fig. 3). This result is in agreement with the observation of Funk and v. Schönborn [1914] that the livers of pigeons fed with a beri-beri-producing diet are poor in glycogen.

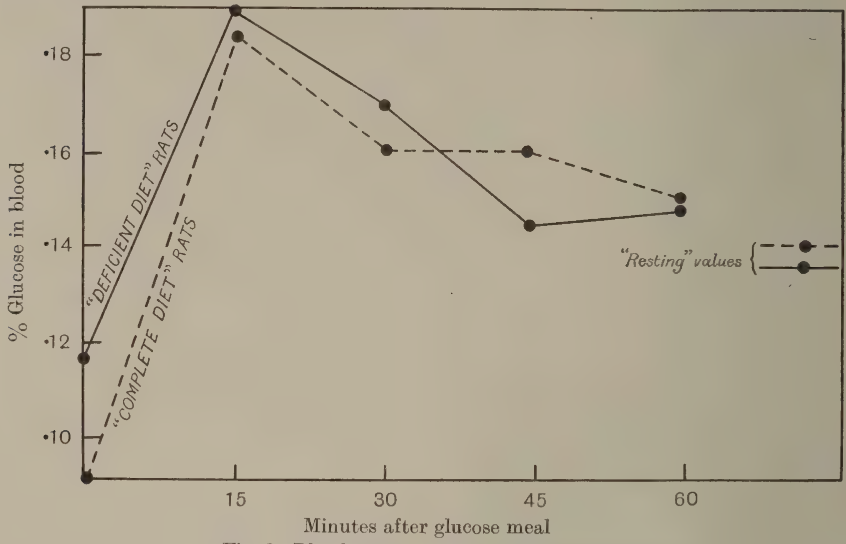

Fig. 2. Blood-sugar curves following glucose meals.

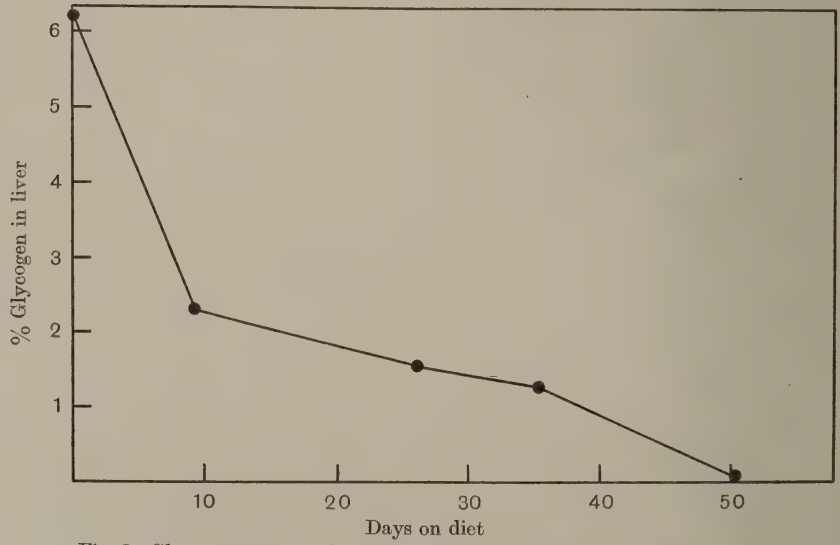

Fig. 3. Glycogen content of livers of rats fed on a vitamin B-deficient diet.

The condition of the vitamin B-deficient rat is in many ways not unlike that producible by underfeeding. Indeed the animal almost invariably is in a fasting condition at the end. A metabolism experiment performed with intent to control this point showed that the vitamin B-deficient rat ate only from 50–60 % of the food taken by animals of the same weight fed with a complete diet. This fall in the food intake occurred practically from the first day on the diet. On the supposition that the insipid taste of the diet (due to its lack of yeast extract) was in part responsible for this lower food intake, an endeavour was made in a parallel experiment to restore the flavour with a meat extract known to be free from vitamin B. This, however, had no appreciable effect. Table II compares the appetites of the rat for the normal control diet (*a*), the vitamin B-deficient diet (*b*), and the latter diet flavoured with meat extract (*c*). Each figure is the mean for three rats over the first 12 days on the diet.

Table II. *Amounts* (*dry weight*) *of food eaten per day per* 100 *g. rat.*

(*a*)	Deficient diet + yeast extract	11·1 g.
(*b*)	Deficient diet	6·3
(*c*)	Deficient diet + meat extract	6·0

It would seem likely from these figures that vitamin B has a direct stimulating influence on the appetite of the rat, apart from other functions. Wright [1921] has shown that the addition of vitamin B to a diet deficient in vitamins A and B (fed to rats) increases peristalsis and stimulates appetite and growth.

SUMMARY.

The normal and basal blood-sugar levels of rats fed on complete and vitamin B-deficient diets have been ascertained.

The rate of absorption of glucose from the alimentary tract has been shown to be unaffected by vitamin B deficiency.

A gradual removal of glycogen from the liver has been shown to follow the elimination of vitamin B from the diet of the rat.

It has been demonstrated that meat extract fails to stimulate the appetite of the rat when substituted for yeast extract in the diet as the sole flavouring principle. It is suggested that there is a causal relationship between the vitamin B content of the diet and the food intake of the rat.

In conclusion we desire to record our gratitude to Prof. J. C. Drummond for much valuable help and encouragement.

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XCV. A NOTE ON THE BASAL VITAMIN B-FREE DIET OF DRUMMOND AND WATSON.

By ALFRED LOUIS BACHARACH.

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(*Received June 25th, 1925.*)

In the testing of food-stuffs and other substances for the growth-promoting water-soluble vitamin, it has been customary to use a basal diet, described by Drummond and Watson [1922], of the following composition:

	Parts
Purified caseinogen (preferably alcohol-extracted)	20
Rice starch	55
Butter fat	15
Salt mixture	5
Orange juice	5

In a test laid down by the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute, this diet has been slightly modified as follows:

	Parts
Caseinogen (vitamin B-free)	20
Rice starch	60
Cod-liver oil	2
Vitamin A-free oil or fat	8
Salt mixture (McCollum's, plus trace KI) ...	5
Lemon juice	5

It was suggested to us by Dr G. A. Hartwell that the presence of lemon juice in this diet may be undesirable, as lemons have been reported to contain appreciable quantities of the vitamin in question. Osborne and Mendel [1920] found vitamin B in "the edible parts of the orange, lemon...," according to the report on Accessory Food Factors (Second Edition, 1924), which gives both orange juice and lemon juice a single + for vitamin B.

A preliminary series of experiments suggested that there was a slight but distinct advantage to be gained by omitting lemon juice from the diet; that no complications would follow this omission was to be expected from the observation, frequently made, that the white rat can thrive for a considerable period in the absence of anti-scorbutic factor.

To settle the matter, 12 young male albino rats from an intensely inbred stock were fed on diets which are made sufficiently clear by reference to Fig. 1.

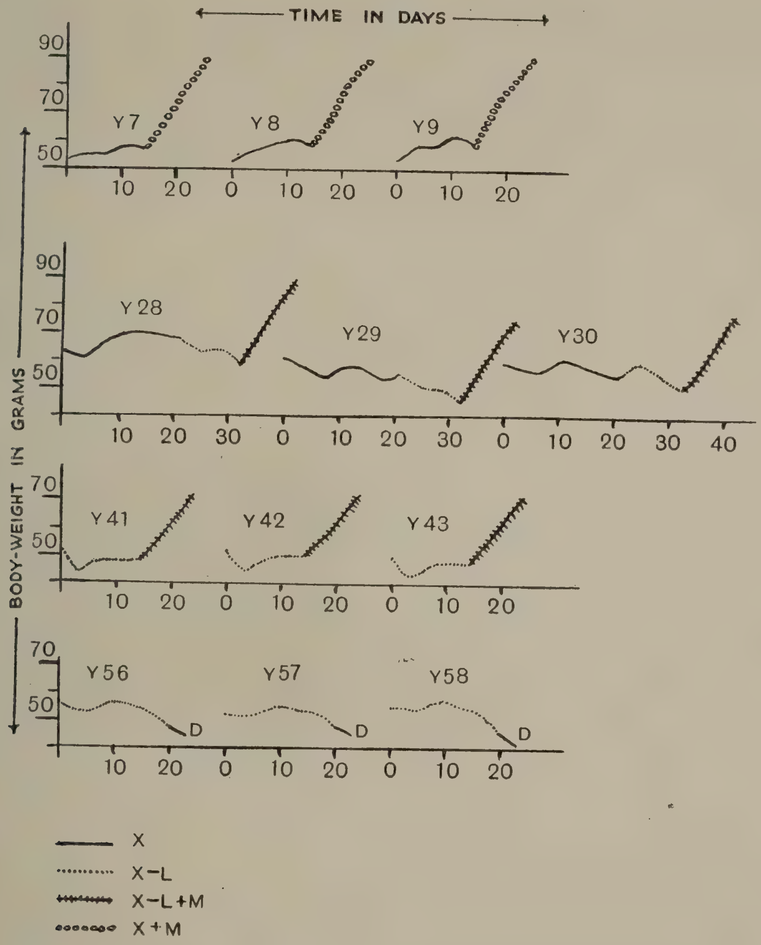

Fig. 1.

X, standard diet.
$X - L$, standard diet with lemon juice omitted.
M, 0·4 g. marmite per animal per day.
D, animal died or was moribund.

The following points emerge from consideration of the growth curves:

(1) Rats on diet X showed distinctly better growth than rats on diet $X - L$.

(2) Rats on diet X for 21 days and then on diet $X - L$ for a further 7 days, though very small and enfeebled, recovered rapidly on administration of vitamin B.

(3) Rats on diet $X - L$ for 20 days were unable, when subsequently fed diet X, to survive more than a further 3 or 4 days.

(4) Rats fed diet $X - L$ for 7 or more days at no time weighed more than 2 g. more than at the beginning of the experiment.

(5) Of six rats on diet X, after 11 days four weighed from 5–10 g. more than at the beginning of the experiment.

(6) Rats which had been on diet $X - L$, when fed vitamin B, recovered very nearly as rapidly as rats on diet X similarly supplemented.

These results appear to be susceptible of two alternative explanations. The omission of lemon juice from the basal diet certainly involves the omission of vitamin C, and possibly involves the omission of vitamin B. It has been shown that the small amount of anti-scorbutic factor (0·5 cc. lemon juice per animal per day) has no effect on the growth of the rat over the periods involved in these experiments [Harden and Zilva, 1918; Drummond, 1919; Parsons, 1920]. It seems, therefore, probable that the explanation of these results lies in the previously established fact that lemon juice contains appreciable amounts of vitamin B. The results also show that, as a practical question of experimental technique, its omission from the diet is desirable and attended by no disadvantages.

SUMMARY.

Cessation of growth of the white albino rat on a diet free from vitamin B can be secured in about 7 days by feeding the basal diet of Drummond and Watson, but without the addition of lemon juice. No disadvantage attends this omission during the experimental period involved.

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XCVI. ON A POSSIBLE RÔLE OF PYRUVIC ACID IN BACTERIAL GROWTH[1].

By JUDA HIRSCH QUASTEL (1851 *Senior Exhibitioner*)

From the Biochemical Laboratory, Cambridge.

(*Received June 19th, 1925.*)

It is interesting to consider the various ways in which the constitution of a synthetic nutrient medium may be changed without appreciably affecting its nutritive character for any particular species of bacterium. It is well known that there are numerous compounds, any one of which, when supplied as the sole source of carbon, will support bacterial growth. A consideration of the various media upon which a particular bacterium will thrive makes it clear that the ability of the organism to grow is in some way connected with the chemical constitution of the substrate. Not all carbon compounds will supply carbon to an organism for growth, and it seems logical to suspect the existence of some relationship between molecules possessing a nutritive character.

If it is assumed from the outset that related to each nutritive molecule there exists a set of *specific* enzymes which bring about various syntheses culminating in the production of protoplasmic material whose nature remains fundamentally the same whatever the nature of the nutrient molecule, it is apparent that an investigation which seeks to determine a relationship between nutrient molecules would be pointless. This assumption, however, is merely a restatement of the fact that nutrition occurs, and since it throws no light on the connection between nutritive character and chemical constitution, and moreover postulates the existence of a great number of specific enzymes for which there is no clear evidence, it cannot be of use in predicting the nutrient character of any given molecule or in interpreting any of the steps by which nutrition occurs. It may be discarded, therefore, in favour of some suggestion which possesses, even in a slight degree, this power of prediction and of interpretation.

Experiment, so far, has failed to demonstrate any fundamental transformation in an organism (say *B. coli* or *B. pyocyaneus*), which is caused by an alteration in the constitution of the nutritive medium. Variations in the strains may occur, but the lack of any fundamental change suggests the presence in any particular organism of an orderly series of reactions which express themselves in growth, multiplication and the general properties which characterise this particular organism. There may be secondary reactions which account for variations in the strains, but the important point is the presence of a definite series of reactions which is characteristic for each bacterial species. If this view is right it is difficult to perceive how two nutrient molecules of widely differing chemical nature (say glutaminic acid and acetic acid) can each support bacterial growth and yet each produce the identical series of reactions which is characteristic of the organism. There appears to be but one explanation—that each nutrient molecule gives rise to a particular substance (which will be termed *P*) which is the first organic material to enter into the synthetic operations of the organism. Thus *P* may be regarded as the initial or primary substrate of the organism, and all other compounds must proceed through *P* before entering into protoplasmic synthesis. It may be that for any organism there exists a number of substances any one of which will act as *P*; but the suggestion which the writer wishes to offer is that this number is strictly limited and may be in many cases simply one. This suggestion does not mean that *P* cannot be the same for any two organisms, for it is clear that subsequent operations on *P* must depend on the nature and variety of enzymes present in the cell, and hence the material into which *P* is built will be characteristic not only of *P* but of these enzymes. It is possible therefore that *P* may be the same for a large number of organisms.

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[1] The views expressed in this communication are contained in a dissertation which was laid before the authorities of Trinity College, Cambridge, in August, 1924.

Only *B. coli* has been studied at all systematically from this point of view, and the evidence points to pyruvic acid (or some very closely allied substance) being the first organic molecule to enter into protoplasmic synthesis. The work of Neuberg and his colleagues gives great prominence to the part played by pyruvic acid in fermentation phenomena and the work of de Graaff and Le Fèvre [1925] supports the view of Neuberg as to the importance of this acid as an intermediate substance during bacterial fermentation. Aubel [1924] has shown that pyruvic acid is an excellent nutrient material for bacteria, and very recently Aubel and Salabartan [1925] have come to the conclusion (from the standpoint of energetics) that in the growth of *B. coli* upon glucose pyruvic acid serves as the source of the bacterial substance. In the work of Quastel, Stephenson and Whetham [1925] on the growth of *B. coli* upon lactate and nitrate, it was shown how the production of nitrite at a toxic concentration afforded an explanation of the fact that much pyruvic acid did not appear in the medium until the nitrite had reached this concentration. "Below this strength of nitrite the pyruvic acid produced from the lactate is almost entirely used for growth. As soon, however, as the nitrite reaches its toxic concentration and prevents further bacterial synthesis the pyruvic acid, which is still being produced from the lactic acid by the action of nitrate and the resting organism, is diverted from synthesis and is liberated from the medium."

The following conclusions follow from the work of Quastel, Stephenson and Whetham [1925] and from the suggestion put forward in this communication.

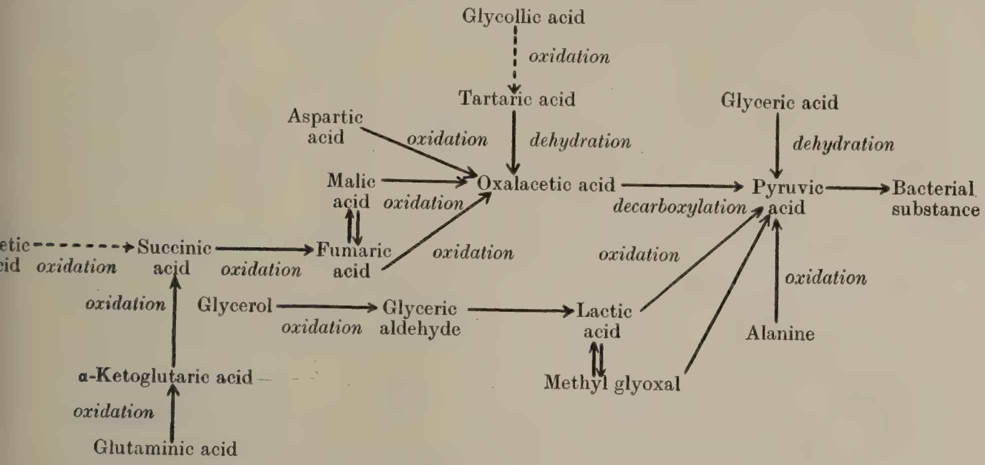
1. That a molecule which is activated by *B. coli*, which can give rise to pyruvic acid and which in its reactions (oxidation, etc.) can liberate energy, may serve as a source of nutrient carbon to *B. coli*. The activation is demon-

strated by the resting bacteria—methylene blue method (see Quastel and Whetham [1925] for details).

2. That a molecule which is not activated by *B. coli* but which can give rise to pyruvic acid cannot serve as a nutrient carbon supply, *e.g.* maleic acid.

3. That a molecule which is activated by *B. coli* but which cannot give rise to pyruvic acid cannot serve as a nutrient carbon supply, *e.g.* formic acid.

The following chart illustrates how various substances can give rise by known reactions to pyruvic acid. All these substances should, if they are activated, serve as nutrient supplies of carbon to *B. coli*.



For experiment a number of substances were investigated and the results are expressed in Table I.

Table I.

Substance	I	II	III	IV
Formic acid	+	0	0	0
Acetic acid	+	+	+	+
Propionic acid	+	0	0	0
Butyric acid	+	0	0	0
Lactic acid	+	+	+	+
α-Hydroxybutyric acid	+	0	0	0
Mandelic acid	+	0	0	0
β-Hydroxybutyric acid	+	0	0	0
Oxalic acid	0	0	0	0
Malonic acid	Slight	0	0	0
Succinic acid	+	+	+	+
Glutaric acid	Slight	0	0	0
Glycol	+	0	0	0
Glycerol	+	+	+	+
Erythritol	0	0	0	0
Fumaric acid	+	+	+	+
Maleic acid	0	+	0	0
Glycine	Slight	0	0	0
Alanine	+	+	+	+
Aspartic acid	+	+	+	+
Glutaminic acid	+	+	+	+
Malic acid	+	+	+	+
Tartaric acid	+	+	+	+
Glyceric acid	+	+	+	+
Glycollic acid	+	+	(+)	(+)

In column I, + shows that the substance is activated by *B. coli*, 0 indicating the absence of activation.

In column II, + shows that the substance may undergo change to pyruvic by reactions of which we are aware (*e.g.* oxidation, dehydration); 0 indicates that it is unlikely that a change to pyruvic acid occurs. In the cases of acetic acid and glycollic acid (which have both been shown by Quastel and Whetham [1925] to be hydrogen donators) it is assumed that the first products of oxidation are succinic and tartaric acids respectively. It is conceivable that propionic acid may undergo a change, similar to that in the body, to glucose or lactic acid, and thence to pyruvic acid, but since there is as yet no direct evidence for this with *B. coli* this assumption will not be made. (β-Hydroxybutyric acid may also be expected to be oxidised through acetoacetic to acetic acid and thence to pyruvic acid, but as yet there is no evidence for this.)

In column III a + sign indicates that the growth of *B. coli* on this substance, present as sole source of carbon under aerobic conditions, is expected; a zero sign has its usual significance. The sign (+) indicates only slight growth.

Column IV gives the experimental results.

For experiment the substance was made up to 0·1 *M* concentration in a solution containing the following:

$$0{\cdot}4\ \%\ (NH_4)_2HPO_4;\ 0{\cdot}1\ \%\ NaCl;\ 0{\cdot}1\ \%\ KH_2PO_4;\ 0{\cdot}07\ \%\ MgSO_4.7H_2O;\ 0{\cdot}5\ \%\ NaNO_3.$$

The media after neutralising to p_H 7·2 were autoclaved in 5 cc. lots and inoculated with two drops of a 20-hour broth culture of *B. coli*. A slight growth generally occurred on the inorganic medium alone, this being due to the growth on the organic matter introduced in the actual inoculation. This growth was taken as a standard zero growth for the comparison of the growths of *B. coli* upon the various media. Growths were examined after 48 hours' incubation at 37°.

In no case so far examined has the experimental result contradicted the one predicted.

It should be added that pyruvic acid may not be the *only* initial or primary substrate; experiment, however, so far does not seem to indicate the existence of another substance which will act in this capacity.

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XCVII. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. II.

By JUDA HIRSCH QUASTEL (1851 *Senior Exhibitioner*)
AND MARGARET DAMPIER WHETHAM.

From the Biochemical Laboratory, Cambridge.

(*Received July 1st, 1925.*)

In Part I of this series [Quastel and Whetham, 1925] an account was given of the behaviour of the following groups of substances as hydrogen donators in the presence of resting *B. coli*.

1. Fatty acids.
2. Saturated dibasic acids.
3. Hydroxy-acids.
4. Polyhydric alcohols.
5. Monohydric alcohols.

A table was given of the relative reducing powers or reducing coefficients of the hydrogen donators.

This communication deals with the behaviour of a number of the amino-acids and the sugars in the presence of resting *B. coli*. The technique adopted throughout the work has been described fully in earlier papers. It consists, briefly, in measuring the rate of reduction of methylene blue under standard conditions of temperature and hydrogen ion concentration, the measurements being made under anaerobic conditions in vacuum tubes.

The experimental results given below are typical examples of a large number of observations. A number of different strains and suspensions of *B. coli* were used and these are referred to as *coli* 1, *coli* 2, etc. The results obtained with *coli* 1 can be compared strictly quantitatively with one another, but only qualitatively with the results obtained with *coli* 2, etc.

The substances under investigation were always brought to a p_H of 7·2.

The Amino-acids.

Glycine appears to be almost inert as a hydrogen donator to methylene blue. It gives indications of a slight activity at very high concentrations but in no case has it been found to produce a complete reduction of the standard quantity of methylene blue used in these experiments. Alanine, on the other hand, is a fairly active donator, its activity being comparable with that of acetic acid. Table I illustrates results with these amino-acids. A succinate reduction is included for comparison.

Table I.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·2, 1 cc. 1/5000 methylene blue solution, 0·5 cc. *coli* 1, and X cc. of the amino-acid solution. The volume of solution in each tube was made up to 6·5 cc. with distilled water. The reduction was carried out *in vacuo* at 45°. A control tube showed no reduction in 6 hours.

Amino-acid	X cc.	Conc. of amino-acid	Reduction time
5 % alanine	1	M/11·6	65 minutes
5 % glycine	1	M/9·7	Not reduced in 6 hours
Succinic acid (control)	—	M/130	10′ 10″

Fig. 1 illustrates the variation in times of reduction of 1 cc. of 1/5000 methylene blue with varying concentrations of alanine at p_H 7·2 and 45° in presence of the same amount of organism.

Leucine gives no sign of reducing activity even at high concentrations.

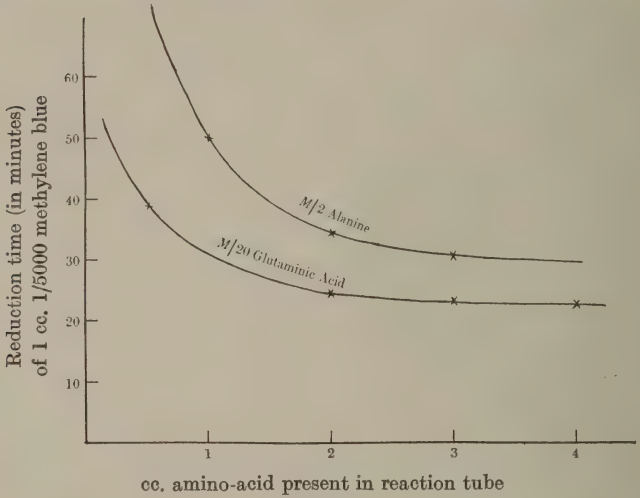

Fig. 1.

Histidine is slightly active at relatively low concentrations, whilst tryptophan is inactive at low concentrations but fairly active at high concentrations (see Table II).

Table II.

Conditions as in Table I.

Amino-acid	Concentration of amino-acid	Organism	Reduction time
Tryptophan	M/130	*Coli* 1	Not reduced in 3 hours
,,	M/26	,, 1	24′ 5″
Leucine	M/17	,, 2	Not reduced in 3 hours
Histidine	M/130	,, 3	169′
,,	M/65·5	,, 3	125′
,,	M/43·3	,, 3	80′
Succinate M/20 (control)	M/65·5	,, 3	5′ 15″

Glutaminic acid appears to be the most active amino-acid yet investigated. Its activity is comparable with that of succinic acid (Fig. 1 gives an example of its reduction time-concentration curve.) Aspartic acid, on the other hand, gives no indication of being a donator of hydrogen but has the properties of a weak hydrogen acceptor. In this respect it resembles malic acid [see Quastel and Whetham, 1924]. Mixtures of succinic acid and aspartic acid, instead of reducing methylene blue completely, reach an equilibrium point which is dependent on the relative concentrations of succinic and aspartic acids. Experimental results with aspartic acid are demonstrated in Table III.

Table III.

Each vacuum tube contained 1 cc. of phosphate buffer p_H 7·2, 1 cc. of 1/5000 methylene blue solution, 2 cc. *coli* 4, 2 cc. of 4 % aspartic acid solution (neutral), and X cc. of $M/20$ sodium succinate. The volume was made up to 7 cc. with distilled water and the reduction carried out *in vacuo* at 45°.

X cc.	Equilibrium point attained in terms of cc. of methylene blue (1/5000)
0·5	0·24
1·0	0·17
1·5	0·10

Aspartic acid is far less active than fumaric acid as a hydrogen acceptor. It is difficult to understand how aspartic acid accomplishes the oxidation of leucomethylene blue unless it undergoes a preliminary deamination to fumaric acid. If this is true the system becomes analogous to the deamination of histidine to urocanic acid [Raistrick, 1917].

It is interesting that the amino-acids which show greatest activity in presence of *B. coli*, viz. glutaminic acid and alanine are also those which show considerable activity in presence of muscle [Thunberg, 1920].

The sugars and related substances.

Glucose appears to be an extremely vigorous donator of hydrogen to methylene blue in presence of *B. coli*. Its activity is greater than that of formic acid (see Table IV).

Table IV.

Each vacuum tube contained 1 cc. phosphate buffer p_H 7·2, 1 cc. of 1/5000 methylene blue solution, 1 cc. *coli* 5, and a quantity of the hydrogen donator. The volume was made up to 7 cc. with distilled water. A control tube did not reduce in 3 hours.

Donator	Concentration of donator	Reduction time
Formic acid	$M/3500$	17′ 30″
,,	$M/1750$	4′
Glucose	$M/3500$	6′ 36″
,,	$M/1750$	5′ 30″

An investigation of the influence of the concentration of the sugars on the reduction time of a quantity of methylene blue shows that at extremely low concentrations the velocity of reduction reaches a value which only very gradually increases with increase in concentration (see Fig. 2). This is typical of the reduction time-concentration curves of most of the sugars.

If glucose donates two atoms of hydrogen per molecule of glucose to methylene blue, then the least quantity of $M/100$ glucose solution which can completely reduce 1 cc. of 1/5000 methylene blue is 0·063 cc. Experiment shows, however, that the least quantity of glucose which will completely reduce 1 cc. of 1/5000 solution of methylene blue (at p_H 7·2 and in presence of *B. coli*) is 0·02 cc. This means that a single molecule of glucose donates at least four (and possibly six) atoms of hydrogen to methylene blue in presence of *B. coli*. The least quantity of formic acid which accomplishes complete reduction of 1 cc. 1/5000 methylene blue solution is 0·1 cc. of a $M/100$ solution, an amount which would be expected if formic acid donated two atoms of hydrogen per molecule.

Laevulose is a good hydrogen donator with *B. coli*, though not so powerful as glucose (see Fig. 2). The lowest concentration of it which reduces 1 cc. of 1/5000 methylene blue solution completely is 0·02 cc. of a $M/100$ solution. Like glucose, therefore, laevulose donates at least four (and possibly six) atoms of hydrogen per molecule. The velocity of reduction due to laevulose at low concentration is less than that due to glucose. A point of practical importance in connection with glucose and laevulose may be mentioned. 0·02 cc. of $M/100$ solution of the sugar corresponds to 0·036 mg. of the sugar which in a volume of 7 cc. readily reduces 1 cc. 1/5000 methylene blue solution; *i.e.* a concentration of glucose corresponding to 1 part in 200,000 may be readily detected by the resting bacteria-methylene blue method.

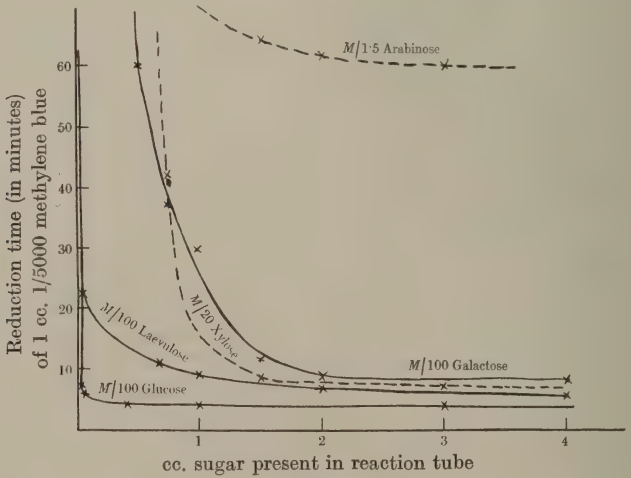

Fig. 2.

Galactose is less active than glucose or laevulose (see Fig. 2) and it is impossible to state by the methylene blue method whether galactose donates more than two atoms of hydrogen per molecule. Mannose is a good donator of hydrogen but this sugar has not yet been examined in detail. It is at least as active as galactose.

Xylose and arabinose, among the pentoses, have been examined. Their effects are indicated in Fig. 2. Arabinose is an extremely poor donator of hydrogen compared with xylose, whilst the latter is not as active as galactose (see Table V).

Table V.

Conditions as in Table IV. *Coli* 6.
A control showed no reduction in 3 hours.

Donator	Concentration of donator	Reduction time
Galactose	*M*/1400	60′
,,	*M*/700	30′
,,	*M*/350	8′ 25″
Maltose	*M*/1400	Not reduced in 2 hours
,,	*M*/280	30′
,,	*M*/140	8′
Arabinose	*M*/21	Not reduced in 2 hours
,,	*M*/10·5	110′
,,	*M*/7	87′
Xylose	*M*/280	Not reduced in 2 hours
,,	*M*/186	41′
,,	*M*/93	8′
Mannitol	*M*/28,000	17′
,,	*M*/35,000	Not reduced in 2 hours
Glucose	*M*/14,000	8′
Laevulose	*M*/14,000	22′
Succinic acid	*M*/700	26′
,,	*M*/70	10′

Among the disaccharides, sucrose appears to be inert. Both lactose and maltose, however, are good hydrogen donators (see Tables V and VI).

Table VI.

Conditions as in Table V. *Coli* 7.
Control showed no reduction in 1 hour.

Concentration of lactose	Reduction time
M/7000	27′ 25″
M/1750	11′ 35″
M/583	9′ 30″

It is difficult, with the disaccharides, to decide how far the reduction may be due to traces of glucose produced by hydrolysis. Harden and Zilva [1915] found no reducing effect with lactose, although a positive effect was obtained with maltose. Under the conditions of our experiments, however, no specimen of lactose has been found to be devoid of reducing activity.

Of the hexahydric alcohols, sorbitol, mannitol and dulcitol have been examined. Both mannitol (see Table V) and sorbitol show considerable reducing activity, whilst dulcitol seems to be practically inert (see Tables VII and VIII).

Mannitol appears to be almost as active as glucose and donates at least four atoms of hydrogen per molecule to methylene blue in presence of *B. coli*.

Only adonitol and arabitol have been examined among the pentahydric alcohols. The former is devoid of any appreciable reducing activity, whilst the latter which was prepared by reduction of arabinose with sodium amalgam seems to be a better donator of hydrogen than arabinose.

Raffinose and rhamnose have been examined and these do not seem to possess any reducing activity.

Table VII.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·2, 1 cc. 1/5000 methylene blue, and the hydrogen donator. Volume made up to 6 cc. with distilled water.

Donator	Concentration of donator	Reduction time
	1 cc. *coli* 8.	
None (control)	—	46′
Glucose	*M*/120	5′ 20″
Mannitol	*M*/120	8′ 15″
Sorbitol	*M*/120	7′ 40″
Saccharic acid	*M*/120	15′ 45″
Succinic acid	*M*/120	5′ 15″
	1 cc. *coli* 9.	
None (control)	—	22′ 30″*
Mannitol	*M*/120	9′ 10″
Sorbitol	*M*/120	9′ 35″
Dulcitol	*M*/120	20′
Adonitol	*M*/120	20′

* *Coli* 9 was a highly reducing organism.

Among the acids corresponding to the sugars, saccharic, gluconic and mucic acids have been investigated only in a qualitative manner. Saccharic and gluconic acids are poor but definite hydrogen donators whilst mucic acid, up to a concentration of *M*/24, is inert.

An examination has been made of several glucosides. Crude α-methylglucoside is a good donator of hydrogen but after being recrystallised a number of times, the pure material is practically devoid of reducing power. So far a specimen of β-methylglucoside has not been obtained which is inert. Amygdalin, if purified by several recrystallisations, is almost inert, although the crude material is nearly as active as glucose. Salicin and phloridzin have not been found to possess any reducing activity.

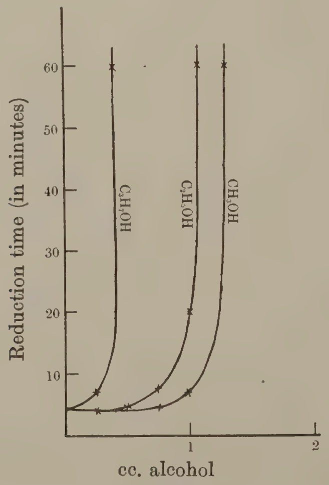

Fig. 3. Effects of methyl, ethyl and propyl alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. *M*/20 glucose in presence of *B. coli*.

The alcohols—methyl, ethyl and propyl—have powerful inhibitory effects on the reduction of methylene blue due to the sugars. The inhibitory effect of methyl alcohol is less than that of ethyl alcohol and this less than that of propyl alcohol (see Fig. 3). The inhibitions are of a similar order to those noted in the case of other hydrogen donators.

The reducing coefficients of some of the amino-acids and sugars with respect to *B. coli* are given below (see Quastel and Whetham [1925] for details with regard to reducing coefficients). They are only to be regarded as approximate and as expressing the correct order of magnitude.

Reducing Coefficients (with respect to B. coli).

Succinic acid	100
Formic acid	700
Alanine	1·0
Glutaminic acid	25
Glycine	less than 0·8
Glucose	5000
Laevulose	5000
Galactose	200
Mannitol	5000
Xylose	20
Arabinose	less than 0·8
Maltose	50

SUMMARY.

An account is given of the behaviour of a number of amino-acids, sugars and related substances in the presence of resting *B. coli* as the activating source and of methylene blue as the hydrogen acceptor. A table is given of the relative reducing powers or reducing coefficients of some of the donators.

We wish to express our appreciation of the interest taken by Sir F. G. Hopkins in this work and the thanks of one of us (M. D. W.) are due to the Medical Research Council for a grant held during this work.

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XCVIII. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. III.

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AND

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(*Received July 1st, 1925.*)

The two previous communications on the activating powers of resting or non-proliferating bacteria were confined exclusively to *B. coli comm.* This paper deals with the activating powers of *B. prodigiosus*, *B. proteus* and *B. faecalis alkaligenes.*

The experimental technique was that adopted in the work on *B. coli* [see Quastel and Whetham, 1924, 1925, 1].

B. prodigiosus.

Resting *B. prodigiosus* was prepared by growing the organism in tryptic broth in Roux bottles for two days at 37°. The organism was centrifuged and washed well with normal saline. The washed *B. prodigiosus* after aeration was made up with normal saline to a suspension of which a suitable quantity (say 0·5 cc.) did not reduce a standard quantity of methylene blue in two hours. To determine the activating powers of this organism (as well as other organisms) a standard series of substances was used. All substances were brought to a p_H of 7·4 prior to investigation. Table I gives the results with *B. prodigiosus*, *B. proteus* and *B. faecalis alkaligenes.*

It will be seen that in practically all respects *B. prodigiosus* is similar to *B. coli comm.* in its dehydrogenating powers. One marked difference appears, however, and this is the effect with glycine. *B. coli* so far has never been found to possess any marked activating power with glycine. As the organism (*B. prodigiosus*) becomes older its activating power on glycine appears to fall off rapidly.

B. proteus.

The organism was prepared in the same way as *B. prodigiosus.*

B. proteus like *B. coli* appears to have no appreciable effect on glycine. It seems to resemble *B. coli* in its dehydrogenating powers. In the presence of high concentrations of the fatty acids *B. proteus*, like *B. coli*, causes rapid reduction of methylene blue. With low concentrations of the fatty acids no decoloration of methylene blue occurs.

Table I.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, a quantity of the suspension of the organism and 1 cc. of a solution of the substance under investigation. The volume of solution in each tube was made up to 6·5 cc. with distilled water. The reduction was carried out *in vacuo* at 45°.

		Reduction time in minutes		
Donator	Concentration of donator	0·5 cc. *B. prodigiosus*	0·5 cc. *B. proteus*	0·2 cc. *B. alkaligenes*
None	Control	Not reduced in 2 hrs.	Not reduced in 2 hrs.	31·0
Formic acid	$M/30$	4·0	2·0	7·25
Acetic acid	$M/13$	32·4		12·9
,,	$M/6{\cdot}5$		2·5	
Propionic acid	$M/13$	64·0		16·1
,,	$M/6{\cdot}5$		16·2	
Butyric acid	$M/13$	74·0		14·9
,,	$M/6{\cdot}5$		90·0	
Isobutyric acid	$M/13$	19·4		22·2
,,	$M/6{\cdot}5$		13·4	
Oxalic acid	$M/13$	Not reduced in 2 hrs.	Not reduced in 2 hrs.	40·0
Malonic acid	$M/13$	,,	,,	40·0
Succinic acid	$M/130$	6·0	5·3	21·0
Glutaric acid	$M/13$	Not reduced in 2 hrs.		
Lactic acid	$M/13$	6·2	5·7	
,,	$M/130$	18·8	13·7	9·0
α-Hydroxybutyric acid	$M/13$	12·0	3·7	4·4
,,	$M/130$	36·0	25·0	
β-Hydroxybutyric acid	$M/13$	4·3	2·4	3·8
,,	$M/130$	3·25	80 % reduced in 90 minutes	
Mandelic acid	$M/13$	15·7	Not reduced in 2 hrs.	14·8
,,	$M/130$	Not reduced in 2 hrs.		26·0
Tartaric acid	$M/13$	27·0	Not reduced in 2 hrs.	32·0
,,	$M/4{\cdot}3$		63·0	
Glycollic acid	$M/13$	Not reduced in 2 hrs.	Not reduced in 2 hrs.	31·0
,,	$M/4{\cdot}3$		90·0	
Glycine	$M/10$	6·0	Not reduced in 2 hrs.	26·0
Alanine	$M/12$	35·8	,,	22·3
Glutaminic acid	$M/19$	15·6		
,,	$M/13$		7·7	12·4
Glycol	$M/13$	51·2	Not reduced in 2 hrs.	31·0
Glycerol	$M/6$	5·6	7·0	29·0
Erythritol	$M/6$	Not reduced in 2 hrs.		
,,	$M/13$		Not reduced in 2 hrs.	31·0

B. faecalis alkaligenes.

The organism was prepared in the same way as the previous two. A marked feature of well-washed *B. alkaligenes* suspensions is the high reducing activity of the organism itself. In this it differs markedly from *B. coli* and *B. proteus*.

It is clear from the results with *B. alkaligenes* that this organism has only a very slight activating power, this power being apparently greatest with the hydroxy-monobasic acids and formic acid (as seems also to be the case with *B. coli*, *B. prodigiosus* and *B. proteus*).

The activations of fumaric, malic and aspartic acids.

There is a marked difference in activating power between *B. alkaligenes* and *B. prodigiosus* or *B. proteus*; the differences, however, between the latter two organisms are not so apparent. Apart from the effect with glycine the differences appear to be simply of a quantitative nature. This quantitative aspect of the differences in activating power between the two organisms is shown most clearly in the case of fumaric acid.

It was shown by Quastel and Whetham [1924] that fumaric acid is activated by *B. coli*, this activation being demonstrated by the power of fumaric acid to oxidise leucomethylene blue only in the presence of the resting organism. *B. prodigiosus* possesses this power of activating fumaric acid to a marked degree; *B. proteus* possesses the same power but apparently to a much smaller extent. It was shown in the case of *B. coli* [Quastel, Stephenson and Whetham, 1925] that activated fumaric acid can play a dual rôle—it can act as a hydrogen acceptor (*e.g.* lactic acid will transfer its hydrogen to fumaric acid in presence of *B. coli*) and it can act as a hydrogen donator (or oxygen acceptor, *e.g.* fumaric acid is oxidised by nitrates in presence of *B. coli*). In the methylene blue experiments with *B. coli*, no reduction of methylene blue by fumaric acid was ever observed. It is clear, however, that if fumaric acid can both accept and donate hydrogen then the reduction velocity of methylene blue in the vacuum tube will be dependent on the velocities of the two reactions which fumaric acid can bring about. (In speaking of fumaric acid as a hydrogen donator the usual nomenclature of reduction phenomena is being adopted. The reduction does not necessarily mean that hydrogen is being transferred from the fumaric acid molecule to methylene blue; it is far more likely in this case that the elements of water play a part in the reaction.)

The rate of reduction of methylene blue by *B. prodigiosus*, like that of *B. coli*, is strongly retarded by fumaric acid. For instance, 2 cc. of a thick suspension of *B. prodigiosus* reduced 1 cc. of 1/5000 methylene blue (without the addition of any donator) in 15 minutes. In presence of fumaric acid (0·8 %) complete reduction was never obtained; the methylene blue was reduced to an equilibrium point which remained stationary. Again, 0·5 cc. of a suspension of *B. prodigiosus*, which did not reduce 1 cc. of 1/5000 methylene blue in two hours, but which in the presence of succinate (0·15 %) reduced in six minutes, did not in the presence of a mixture of succinate (0·15 %) and fumarate (0·8 %) bring about a complete reduction of the methylene blue after several hours; an equilibrium point was reached. Just as with *B. coli*, therefore, *B. prodigiosus* strongly activates fumaric acid, so that the velocity with which the latter oxidises leucomethylene blue is greater than that with which reduction of the dyestuff occurs. It is possible by using a sufficiently large amount of organism in the presence of an active donator to produce an extremely rapid rate of reduction of methylene blue—this reduction being so rapid that even if fumaric acid is present the oxidising power of the latter does not exhibit itself until after a few minutes, when possibly all the methylene blue has been reduced. For instance, 2 cc. of a thick suspension of *B. prodigiosus* reduced 1 cc. of 1/5000 methylene blue solution in 15 minutes; in presence of succinate (0·15 %) it reduced in five minutes; in presence of a mixture of succinate (0·15 %) and fumarate (0·8 %) it reduced the methylene blue almost to completion in six minutes and then oxidation of the leucomethylene blue commenced, for the colour of the solution instead of disappearing completely quickly became bluer until it reached an intensity which remained stationary for several hours. This phenomenon will be referred to as "reversal." (Reversal was first noticed by Quastel and Whetham in the case of *B. coli* and β-hydroxybutyric acid and will be discussed in detail elsewhere.) If a sufficiently large quantity of *B. coli* be used, reversal can be demonstrated in a mixture of succinate and fumarate, just as with *B. prodigiosus*. In the absence of an active donator such as succinate neither *B. coli* nor *B. prodigiosus* has been found to exhibit reversal with fumaric acid. This demonstrates clearly the high oxidising power of the latter acid in presence of these organisms.

With *B. proteus* an activation of fumaric acid is produced; but in this case reversal can be observed with fumaric acid alone. For instance, 1 cc. of a *B. proteus* suspension did not alone reduce 1 cc. of 1/5000 methylene blue in two hours; in presence of fumarate (0·8 %) it reduced practically to completion in six minutes and then reversal occurred, the colour of the solution becoming deep blue by the end of two hours. In the presence of succinate (0·15 %) it reduced completely in four minutes and in the presence of both succinate (0·15 %) and fumarate (0·8 %) a complete reduction occurred in eight minutes, a slight reversal only being observed after a considerable time. It is clear, therefore, that in the case of *B. proteus* the velocity with which fumaric acid oxidises leucomethylene blue is small compared with that in the case of *B. prodigiosus*—or of *B. coli*. This difference is well illustrated by the results given in Table II.

Table II.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, 0·5 cc. of the resting organism, 1 cc. $M/20$ succinate and X cc. of 5 % fumarate. The solution was made up to 6·5 cc. with distilled water and the reduction carried out *in vacuo* at 45°. Controls (in the absence of succinate or fumarate) showed no reduction in two hours.

Organism	X cc.	Observation
B. prodigiosus	0	Reduced completely in 6 mins.
,,	0·1	Stationary equilibrium point reached after 19 mins. Complete reduction not attained. No reversal observed
,,	0·2	Equilibrium attained. No complete reduction. No reversal
,,	0·4	Do.
,,	0·8	Do.
B. proteus	0	Reduced completely in 6 mins. 25 secs.
,,	0·1	Reduced completely in 8 mins. No reversal observed after 1 hour
,,	0·2	Reduced to completion in 10 mins., and then slight reversal
,,	0·4	Reduced almost to completion and then reversal
,,	0·8	Do.

With malic and aspartic acids similar effects appear. It was shown in the case of *B. coli* that both malic and aspartic acids have the effects of weak hydrogen acceptors [see Quastel and Whetham, 1924; 1925, 2]. *B. prodigiosus* behaves similarly to *B. coli*. For instance, 1 cc. of a thick suspension of *B. prodigiosus* alone reduced 1 cc. 1/5000 methylene blue solution in 14 minutes, whilst in presence of malate (0·8 %) complete reduction was not observed but an equilibrium point was attained; in the presence of aspartate (0·8 %) there was reduction almost to completion followed by reversal. With succinate (0·15 %), the presence of malate (0·8 %) retarded the velocity of reduction, reversal not being observed after one hour. Aspartic acid (0·8 %) had no appreciable effect on the velocity of reduction due to succinate. In the case of *B. proteus* there appeared to be no marked effects on malic or aspartic acid.

B. alkaligenes showed no appreciable activating power on fumaric, malic or aspartic acid.

The effect of malonic acid.

It was observed by Quastel and Whetham [1925, 1] that malonic acid had a retarding effect on the velocity of reduction of methylene blue by *B. coli* in the presence of succinate. Neither oxalic nor glutaric acid has such an effect. *B. prodigiosus* and *B. proteus* have now been examined and malonic acid again shows the same anomalous behaviour (see Table III).

Table III.

Conditions as in Table II, but with the addition of 1 cc. of $M/2$ oxalic or malonic acid instead of X cc. of fumaric acid.

Organism	Acid	Reduction time
B. prodigiosus	None (control)	6 mins. 5 secs.
,,	Oxalic	6 ,, 5 ,,
,,	Malonic	18 ,, 43 ,,
B. proteus	None (control)	6 ,, 20 ,,
,,	Oxalic	8 ,,
,,	Malonic	Not completely reduced in 1 hour

Table IV.

Conditions as in Table II, but with 1 cc. of 5 % KNO_3 or 1 cc. $M/10$ $KClO_3$ instead of X cc. of fumarate.

Organism	Oxidant	Reduction time
0·5 cc. *B. prodigiosus*	None (control)	6 mins.
,,	Nitrate	Not reduced after 2 hrs.
,,	Chlorate	,,
0·5 cc. *B. proteus*	None (control)	3 mins.
,,	Nitrate	Not reduced after 2 hrs.
,,	Chlorate	,,
0·2 cc. *B. alkaligenes*	None (control)	20 mins.
,,	Nitrate	15 ,,
,,	Chlorate	16 ,,

Activations of nitrates and chlorates.

Like *B. coli* [see Quastel, Stephenson and Whetham, 1925] both *B. prodigiosus* and *B. proteus* strongly activate nitrates and chlorates, so that these are able to oxidise leucomethylene blue in presence of the organism. *B. alkaligenes* appears to have no appreciable activating power on nitrates and chlorates. Table IV illustrates the experimental results.

DEMONSTRATION OF THE THERMOLABILITY OF THE MECHANISMS DEALING WITH FUMARATE, NITRATE AND CHLORATE.

The demonstration can be carried out by means of vacuum U-tubes [see Quastel and Whetham, 1924], but the following experiments, which are dependent on the fact that *B. alkaligenes* does not activate (to any appreciable extent) nitrate, chlorate or fumarate, are equally convincing. Three solutions are made up; the first containing *B. alkaligenes* (which itself reduces methylene blue very quickly) and, say, the nitrate; the second containing *B. alkaligenes*, *B. prodigiosus* heated for five minutes at 100°, and the nitrate; and the third *B. alkaligenes*, *B. prodigiosus* unheated, and the nitrate. The effects of these solutions on the reduction of methylene blue are observed. It is found that the solution containing the *B. alkaligenes* alone and that containing the mixture of *B. alkaligenes* and heated *B. prodigiosus* reduce the methylene blue in approximately the same time, whilst that containing the unheated *B. prodigiosus* remains blue permanently. Table V gives a complete set of results.

Table V.

Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue, 0·5 cc. *B. alkaligenes* suspension, 1 cc. of *B. prodigiosus* or *B. proteus* (heated or unheated), 1 cc. of the oxidant. The volume was made up to 6·5 cc. with distilled water and reduction carried out *in vacuo* at 45°.

B. prodigiosus		*B. proteus*			
Unheated	Heated	Unheated	Heated	Oxidant	Reduction time
—	—	—	—	None (control)	4 mins. 30 secs.
—	—	—	—	$M/10$ $KClO_3$	4 mins. 30 secs.
1 cc.	—	—	—	,,	Not reduced after 2 hrs.
—	1 cc.	—	—	,,	4 mins.
—	—	1 cc.	—	,,	Not reduced after 2 hrs.
—	—	—	1 cc.	,,	3 mins. 13 secs.
1 cc.	—	—	—	5 % KNO_3	Not reduced after 2 hrs.
—	1 cc.	—	—	,,	4 mins. 13 secs.
—	—	1 cc.	—	,,	Not reduced after 2 hrs.
—	—	—	1 cc.	,,	6 mins.
1 cc	—	—	—	5 % fumarate	Reduction in 6 mins. 20 secs. followed by strong reversal
—	1 cc.	—	—	,,	Reduction in 6 mins. 30 secs. *not* followed by any reversal
—	—	1 cc.	—	,,	Reduction in 2 mins. followed by reversal
—	—	—	1 cc.	,,	Reduction in 9 mins. *not* followed by reversal

The activation of the sugars.

The reducing power of a number of the sugars and the corresponding alcohols in the presence of the three organisms discussed in this paper have been examined. Table VI illustrates the results.

Table VI.

Conditions as in Table I, but with different organisms.

Sugar (etc.) concentration = $M/130$	Reduction time with 0·5 cc. *B. prodigiosus*	Reduction time with 0·5 cc. *B. proteus*	Reduction time with 0·2 cc. *B. alkaligenes*
None (control)	Not reduced in 2 hrs.	Not reduced in 2 hrs.	24 mins.
Glucose	5 mins. 4 secs.	14 mins. 49 secs.	22 ,,
Laevulose	5 ,, 53 ,,	16 ,, 6 ,,	18 ,,
Mannose	7 ,, 40 ,,	Not reduced in 2 hrs.	23 ,,
Galactose	4 ,, 42 ,,	,,	20 ,,
Sucrose	33 ,, 42 ,,	,,	18 ,,
Lactose	11 ,, 15 ,,	,,	19 ,,
Maltose	9 ,, 12 ,,	,,	12 ,,
Xylose	10 ,, 58 ,,	,,	16 ,,
Arabinose	Not reduced in 1 hr.	,,	35 ,,
Sorbitol	10 mins. 2 secs.	,,	24 ,,
Mannitol	8 ,, 9 ,,	,,	26 ,,
Dulcitol	44 ,,	,,	19 ,,

The effects on the sugars may be summarised briefly:

1. *B. prodigiosus* activates powerfully all the compounds investigated, with the exception of dulcitol, sucrose and arabinose which it activates only slightly. It resembles therefore *B. coli* [see Quastel and Whetham, 1925, 2] which, however, does not activate dulcitol or sucrose to any appreciable extent.

2. With the exception of glucose and laevulose *B. proteus* appears not to activate the sugars to any considerable extent.

3. *B. alkaligenes* has a slight effect with maltose but none appreciably on the other sugars.

SUMMARY.

An account is given of the activating powers of *B. prodigiosus*, *B. proteus* and *B. faecalis alkaligenes*.

1. *B. alkaligenes* shows only feeble activating powers, reducing effects (with methylene blue as hydrogen acceptor) being greatest with formic, lactic, α- and β-hydroxybutyric acids. No oxidising action of fumarates, malates, aspartates, nitrates or chlorates can be demonstrated with this organism. Its power of activating the sugars is also very slight.

2. *B. prodigiosus* possesses powerful activating properties, which appear in the cases of fumarates, malates and aspartates to be greater than those of *B. coli*. It activates all the sugars tested, its effects with sucrose, arabinose and dulcitol being relatively weak.

3. *B. proteus* is less powerful than *B. prodigiosus* or *B. coli* in activating fumarates, malates or aspartates as hydrogen acceptors. Its action on nitrates

and chlorates is similar to that of these organisms. Only glucose and laevulose, among the sugars, appear to be activated by *B. proteus* to any considerable extent.

4. The thermolability of the mechanisms in *B. prodigiosus* and *B. proteus* which activate fumarates, nitrates and chlorates is demonstrated.

Our thanks are due to Sir F. G. Hopkins for the interest he has taken in this work.

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XCIX. FURTHER OBSERVATIONS ON THE ANAEROBIC GROWTH OF BACTERIA.

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(*Received July 1st, 1925.*)

In a previous paper [Quastel, Stephenson and Whetham, 1925] the view was advanced that anaerobic growth of an organism might be expected to occur on a pair of organic compounds *A* and *B* (neither of which supports anaerobic growth alone) when these fulfil the following conditions:

(1) That both *A* and *B* are "activated" by the organism so that simultaneous oxidation and reduction may occur.

(2) That the energy necessary for growth is liberated in the interaction.

(3) That as a result of such an interaction some substance is produced capable of entering into the synthetic processes of the cell.

It was then shown that these conditions were fulfilled in the case of *B. coli communis* by lactic acid and nitrate, and by lactic acid and fumarate, the activations of these substances by the organism having been demonstrated by the methylene blue technique. The lactate behaves as a hydrogen donator and the nitrate and fumarate as hydrogen acceptors. It was noted that the interaction of both pairs of substances occurred with output of energy. No anaerobic growth was obtained on lactate alone or on fumarate alone. In this communication an attempt has been made to extend these observations, in the first place, so as to include the growth of *B. coli* on other pairs of substances (which have been shown to be activated) and in the second place to compare the results obtained with *B. coli* with those of other organisms possessing different activating powers. Glycerol, for example, is activated by *B. coli* [Quastel and Whetham, 1925, 1] and both glycerol and nitrate and glycerol and fumarate, if they interact, do so with output of energy, *e.g.*

$$\mathrm{CH_2(OH).CH(OH).CH_2.OH + COOH.CH:CH.COOH}$$
$$\mathrm{= CH_3.CH(OH).COOH + COOH.CH_2.CH_2.COOH + 33\ Cal.}$$

and

$$\mathrm{CH_2(OH).CH(OH).CH_2.OH + 2COOH.CH:CH.COOH}$$
$$\mathrm{= CH_3.CO.COOH + 2COOH.CH_2.CH_2.COOH + 50\ Cal.}$$

Experiment shows that whilst *B. coli* does not grow anaerobically on glycerol alone or on fumarate alone it grows luxuriantly under anaerobic conditions on a mixture of glycerol and nitrate or of glycerol and fumarate.

Table I.

	Fumaric acid			Malic acid			Aspartic acid			Nitrates			Oxygen		
	Coli	*Proteus*	*Prodig.*	*Coli*	*Proteus*	*Prodig.*	*Coli*	*Proteus*	*Prodig.*	*Coli*	*Proteus*	*Prodig.*	*Coli*	*Proteus*	*Prodig.*
Lactic acid	+	0	+*	0	0	(+)*	?	0	0	+*N*	+*N*	+*N*	+	?	+
Glycerol	+	0	+*	+	0	(+)*	+	0	(+)*	+	+	+*N*	+	0	+
Glutaminic acid	0	0	+*	0	0	+*	0	0	(+)*	+	+	+	(+)	(+)	+
Succinic acid	0	0	(+)*	0	0	0	0	0	0	+	(+)	(+)	+	(+)	+
Acetic acid	0	0	0	0	0	0	0	0	0	+	(+)	+	+	(+)	+
Alanine	0	0	(+)*	0	0	0	0	0	0	0	0	0	(+)	(+)	+
Fumaric acid	0	0	+*	0	0	+*	0	0	+*	+	+	+*N*	+	(+)	+
Malic acid	0	0	+*	0	0	+*	0	0	(+)*	+	+*N*	+	+	(+)	+
Aspartic acid	0	0	+*	0	0	(+)*	0	0	(+)*	+	+	+*N*	+	(+)	+

Experiments have been made with a series of substances acting as hydrogen donators coupled with a series of substances acting as hydrogen acceptors with a view to determining their ability to support anaerobic growth. The inorganic medium employed was the same as that adopted in our previous work [Quastel, Stephenson and Whetham, 1925]. 0·5 % of each of the carbon compounds was added to the inorganic medium and the p_H adjusted to 7·4. Organic acids were added as their sodium salts. 5 cc. of medium were placed in each test-tube and inoculated with 0·1 cc. of a 24-hour broth culture. Growths were examined after 48 hours' anaerobic incubation at 37°. The activations of these substances by the organisms in question are dealt with in another paper [see Quastel and Wooldridge, 1925]. The growth obtained on pairs of donators and acceptors appears in Table I. On pairs of donators only no growth was obtained in any case, the donators examined being lactic acid, glycerol, alanine, acetic acid and glutaminic acid. Each entry in the table represents a medium whose carbon is represented by those compounds found in the corresponding abscissa and ordinate. A + sign indicates good growth; a (+) sign indicates a definite but feeble growth; a +* sign indicates anomalous behaviour which will be discussed later; a letter *N* indicates the production of pyruvic acid as indicated by the characteristic nitroprusside reaction.

Discussion of Results.

Anaerobic growth of B. coli *with malic and aspartic acid as hydrogen acceptors.*

Quastel and Whetham [1924, 1925, 1] have found that in the presence of resting *B. coli* malic and aspartic acids act as weak hydrogen acceptors. (Harden's observation [1901] that aspartic acid in the presence of *B. coli* growing in a sugar solution is completely reduced to succinic acid demonstrates the ease with which this compound undergoes reduction.) Hence aspartic and malic acids might be expected to replace fumaric acid as hydrogen acceptors and to support anaerobic growth in presence of suitable donators. Experiment showed, however, that no growth was obtained on lactate and malate and the growth on lactate and aspartate was doubtful. This result corresponds to the weak action of these substances as hydrogen acceptors compared with fumaric acid. When, however, lactate was replaced by another hydrogen donator, glycerol, growth was obtained in all three cases, *i.e.* with fumaric, or malic or aspartic acid; no growth was obtained on any other combination of these substances.

So far no hydrogen donator has been found to replace lactic acid or glycerol satisfactorily as the accessory substance to fumaric acid in supporting the anaerobic growth of *B. coli*. Succinic, acetic and glutaminic acids and alanine all act as donators of hydrogen in the presence of *B. coli* but all fail to support growth in the presence of fumarate as a hydrogen acceptor. This may be due to the energy liberated being insufficient for the purposes of growth or the explanation may be that the products of interaction fail to produce the substance or substances necessary for cell synthesis (for example, pyruvic acid [see Quastel, 1925]).

Correlation of the lack of activating power of B. alkaligenes *with its failure to grow anaerobically.*

The results in a previous communication [Quastel and Wooldridge, 1925] have shown that *B. alkaligenes* cannot appreciably activate nitrates or fumarates. This renders it improbable that anaerobic growth on these substances will occur since they will be unable to act as hydrogen acceptors. Experiment shows that this organism fails to grow anaerobically in the presence of nitrates or fumarates on lactic acid, glycerol, glutaminic acid, succinic acid or acetic acid. Neither will it grow anaerobically on glucose nor on tryptic broth, though aerobically it grows on both of these. The strictly aerobic character of this organism agrees entirely with its lack of activating power towards hydrogen acceptors.

Correlation of the activating power of B. proteus *towards hydrogen acceptors with its restricted powers of anaerobic growth.*

The results obtained in the vacuum tube show that *B. proteus* readily activates nitrates, that it activates fumarates (as compared with *B. coli*) to a relatively slight extent and that its activation of malate and aspartate as hydrogen acceptors is negligible. Corresponding with these observations it is found that the only hydrogen acceptor on which the anaerobic growth of this organism takes place is nitrate.

Correlation of the activating power of B. prodigiosus *with its anaerobic growth.*

B. prodigiosus, having been found to activate all four hydrogen acceptors, viz. nitrate, fumarate, malate and aspartate, it would be expected that anaerobic growth of this organism might occur on any of these substances in the presence of a suitable hydrogen donator. In the presence of lactic acid or glycerol, positive results have, in fact, been obtained, but these do not occur with complete regularity, and appear to be complicated by other factors requiring special consideration.

It appears, for example, that a slight anaerobic growth occurs also on tubes containing fumarate, malate or aspartate alone, though such growths are not as heavy as those usually obtained when glycerol is also present. From the results obtained with the vacuum tube it seems that *B. prodigiosus* activates nitrates, fumarates, malates and aspartates more vigorously than does *B. coli*, and hence should prove to be an equally successful facultative anaerobe. We have found that on inoculating 0·1–0·2 cc. of a broth culture of this organism into 5 cc. of inorganic medium containing 0·5 % potassium nitrate, but no additional source of carbon, a definite growth was obtained. The most probable explanation of this seems to be that the highly activated nitrate behaves as a vigorous hydrogen acceptor to the constituents of the broth introduced with the inoculation, and that growth is thereby rendered possible. As fumarate is vigorously activated by this organism it seems possible that its oxidising action may approach that of nitrate and that the anaerobic growth found on fumarate alone may also be due to its acting as an acceptor to some constituents of the broth. Such a view is strengthened by the evidence obtained by change of p_H. Whenever growth occurs on fumarate, or other dicarboxylic acid, as sole source of carbon (*i.e.* aerobically, or in presence of nitrate) the p_H of the medium changes to the alkaline side (*i.e.* above p_H 8) due to the decarboxylation of the oxidised acid. In cases where anaerobic growth of *B. prodigiosus* apparently occurred on fumarate (or malate or aspartate) alone, no such change to alkalinity was observed. This would indicate that the fumaric acid was acting solely as a hydrogen acceptor and that the material for synthesis was being provided by the broth, introduced with the inoculation. An attempt was made to verify this by sowing tubes containing fumarate alone, or fumarate and glycerol, with saline emulsions of *B. prodigiosus* taken from agar slopes. No growth, however, appeared anaerobically on these media, although good growth occurred aerobically. *B. coli*, on the other hand, developed equally well anaerobically on a glycerol-fumarate medium whether sown from broth or from a saline emulsion taken from an agar slope. We are therefore unable to state with certainty whether the small growths obtained anaerobically with *B. prodigiosus* on fumarate alone (and to a smaller extent on malate or aspartate) are to be attributed to the action of these substances as hydrogen acceptors towards the broth introduced with the inoculation, or to other unexplained causes. In other respects the anaerobic growth of *B. prodigiosus* accords with its activating powers.

ANAEROBIC GROWTH ON PYRUVIC ACID.

Repeated experiments with *B. coli* and *B. prodigiosus* have shown that with large inoculations, these organisms will develop anaerobically on pyruvic acid as sole source of carbon. This confirms the observation of Aubel and Salabartan [1925] and is a correction of a statement made by us in our previous communication [Quastel, Stephenson and Whetham, 1925]. With small inoculations from a fresh culture, or with large inoculations from an older culture, growth may be altogether absent or late in appearing, and we attribute our first negative observation to these facts. The anaerobic growth obtained, however, is small compared with that occurring in the presence of nitrate or air. (This is especially the case with *B. prodigiosus*.) It is, of course, difficult to be certain that the pyruvic acid does not contain traces of other substances which may be playing a part; we have used media which have been sterilised by filtration through a candle and by autoclaving and have obtained small growths in both cases. Assuming the growth to occur on pyruvic acid alone the necessary energy for synthesis may be obtained by a Cannizzaro reaction thus:

$$CH_3 . CO . COOH + H_2O + CH_3 . CO . COOH = CH_3 . CH(OH) . COOH + CH_3 . COOH + CO_2 + 15 \text{ Cal.}$$

It is noteworthy that the anaerobic growth of *B. coli* on 1 % pyruvic acid can be completely inhibited by 0·5 % formic acid (as sodium formate). The formate has no effect, however, on the aerobic growth of *B. coli* on pyruvic acid nor on its anaerobic growth on pyruvic acid in the presence of 0·5 % nitrate. It seems probable, therefore, that the inhibitory effect of formic acid is due to a reducing action which is neutralised by the presence of air or nitrate.

THE EFFECT OF NEUTRAL SALTS AND SALTS OF ORGANIC ACIDS ON ANAEROBIC GROWTH.

The development of *B. coli* anaerobically on lactate and fumarate is powerfully affected by the relative concentrations of these substances. Thus it is shown in Table II that increasing the concentration of lactate relative to fumarate results in inhibition, whereas the reverse procedure has no effect.

Table II.

Lactate %	0·5	0·5	0·5	0·5	1·0	1·25	1·5
Fumarate %	0·5	0·75	1·0	1·5	0·5	0·5	0·5
Growth (48 hrs.)	+ +	+ +	+ +	+ +	+ +	(+)	?

Table III shows that if varying concentrations of succinate be added to the medium growth may be correspondingly decreased and that the inhibitive effect of the succinate is greater in a 0·5 % concentration of lactate and fumarate than if this is raised to 1 %.

Table III.

Lactate %	0·5	0·5	0·5	0·5	0·5	1·0	1·0	1·0	1·0	1·0
Fumarate %	0·5	0·5	0·5	0·5	0·5	1·0	1·0	1·0	1·0	1·0
Succinate %	0	0·2	0·5	0·75	1·0	0	0·75	1·0	1·5	2·0
Growth (48 hrs.)	+ + +	+ + +	+ +	+	0	+ + +	+ + +	+ +	+	0

Table IV shows the effect of adding sodium chloride solution to a lactate-fumarate medium; the results indicate that the inhibitive effect of salts of organic acids is not entirely attributable to osmotic effects.

Table IV.

Lactate %	0·5	0·5	0·5	0·5
Fumarate %	0·5	0·5	0·5	0·5
*NaCl %	0	0·5	0·75	1·0
Growth	+ +	+ +	+ +	+(+)

* The concentration of NaCl does not include that present in the original inorganic medium

Table V shows the relative effects of tartrate and erythritol.

Table V.

Lactate %	0·5	0·5	0·5	0·5	0·5	0·5
Fumarate %	0·5	0·5	0·5	0·5	0·5	0·5
Tartrate %	0	0·25	0·5	0·75	0	0
Erythritol %	0	0	0	0	0·5	1·0
Growth	+ +	+	0	0	+ +	+(+)

It is noteworthy that although salts of organic acids generally have a considerable inhibitive power on the anaerobic growth of *B. coli*, erythritol appears to have little or none.

It would be unprofitable at present, however, to discuss further or to advance any theory to explain these effects. They are appended to indicate the caution necessary in the interpretation of results where such factors may be playing a part.

SUMMARY.

The anaerobic growth of certain bacteria on synthetic media has been further correlated with the activating powers of the organism in question.

Anaerobic growth has only been found possible where the organism activates some constituent of the medium as a hydrogen acceptor.

B. alkaligenes, which is unable to activate nitrate or fumarate, is incapable of anaerobic growth in the presence of these substances.

B. proteus can activate nitrate; its power to activate fumarate as a hydrogen acceptor is however weak in comparison with that of *B. coli* and *B. prodigiosus*. In agreement with these observations it is found that *B. proteus* can grow anaerobically in the presence of nitrate and certain hydrogen donators, but not in the presence of fumarate and these donators.

B. coli and *B. prodigiosus*, which both activate nitrate, fumarate and also malate and aspartate, can grow anaerobically on any one of these substances with glycerol (or in some cases lactate) as a hydrogen donator.

Some effects of salts of organic acids on the anaerobic growth of *B. coli* on fumarate and lactate are noted.

The thanks of one of us (M. S.) are due to the Medical Research Council for a grant which enabled her to pursue this investigation. The thanks of both of us are also due to Sir F. G. Hopkins, for his continued interest in this work.

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C. CHOLESTEROL SECRETION IN THE URINE. PART I.

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Report to the Medical Research Council.

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According to the view most commonly held at the present time, the liver is the organ chiefly concerned with the excretion of cholesterol (exogenous and endogenous). But, in addition to the excretion in the bile, loss occurs in the secretion of sweat and sebum in man, and in the wool fat in sheep; considerable amounts also are eliminated in milk. The kidney has not generally been considered an organ concerned with the excretion of cholesterol, though for a long time it has been known that normal urine contains traces of cholesterol and that this is increased sometimes in pathological conditions.

Bacmeister and Havers [1914] and Pribram [1906] reported only traces in normal urine. More extended investigations were made by Gérard [1911], who found 11 mg. of cholesterol in 70·9 litres of normal urine, which, reckoning the daily urine output as 1500 cc., would be an excretion of 0·23 mg. per day. In pathological cases, Kaiserling-Orgler [1902] and F. Munk [1913, 1916, 1918] reported the presence of doubly refracting lipoids in the urine in cases of renal disease. These were, however, only microscopical examinations. Gross [1921] by chemical means detected the presence of cholesterol in urine in kidney diseases and Bauman and Hausmann [1920] made quantitative estimations of neutral fat and cholesterol in a case of "lipo-lipoiduria" in a nephritic. Falk and Siebenrock [1911] failed to find anisotropic substances in the urine in diabetes mellitus but found considerable quantities in a case of diabetes complicated with nephritis. Genck [1918] found no doubly refracting substances, that is cholesterol esters, in the urine sediment from five diabetic patients. M. B. Schmidt [1921] reported the presence of cholesterol in the urine in a case of acute yellow atrophy of the liver in which the kidney showed marked fatty change: the blood contained 0·24 % of cholesterol and the post-mortem urine 32·35 mg. per 100 cc. Grunke [1922] using Weston's modification of Autenrieth and Funk's method found in normal urine only traces of cholesterol of the order of 1 mg. per day; out of nine cases of icterus, in only one case did he find more than traces, viz. 10·1 mg. of cholesterol per day with a serum cholesterol content of 0·182 %; of five diabetic urines one only showed more than traces, viz. 12·9 mg. per day with a serum content of 0·236 %. The urine sediments according to Grunke were too sparing to be regarded as a source of the cholesterol found; on the other hand, the cholesterol blood pictures suggested no relationship with the cholesterol excreted by the kidney.

The discovery by one of us [Gardner, 1924] in the bile of the hippopotamus of a small quantity of a cholesterol compound not hydrolysable by alkalies but hydrolysable by strong acids, which substance it was thought might be an ethereal sulphate, suggested the possible presence of this body in the urine and the possibility of correlating this with the blood picture in cases of marked hypercholesterolaemia.

We therefore examined normal urines taken from healthy students, and also urines obtained from pathological cases.

Method used.

The urine was very carefully filtered to make sure that cell debris was got rid of, and was then made up, sometimes after partial concentration, to 2 % content of caustic soda and thoroughly extracted with ether. To do this the urine was treated with one-half of its bulk of ether, repeatedly shaken during the day, allowed to stand over night, then separated and re-extracted with fresh ether in a similar way during four or five days, until we were quite certain that all the cholesterol was extracted. The ethereal solution was evaporated, the residue hydrolysed by alcoholic solution of sodium ethoxide and the cholesterol estimated by digitonin, using the procedure of Frazer and Gardner [1910]. The extracted urine was next evaporated to dryness and the residue boiled with glacial acetic acid under a reflux for 5 to 6 hours. The fluid was then diluted with water, the acetic acid neutralised with strong caustic soda and then made alkaline. The alkaline fluid was thoroughly extracted as before, the ethereal solution evaporated and the residue boiled with alcoholic sodium ethoxide to hydrolyse any cholesterol acetate, and the cholesterol then determined as before.

The results are summarised in Table I.

It will be seen that normal urine contains a measurable amount of cholesterol, partly in the form of either free or ester cholesterol and partly in the form of some compound hydrolysable by strong acid and not by alkalies. We are unable to say definitely what this compound is, but the following facts—that it is not hydrolysable by alkalies, but is hydrolysable only by strong acid, that it appears to be in a very stable condition as it does not decompose during evaporation and that it remains in solution after coagulation of the proteins in a highly albuminous urine—suggest that it is sodium or potassium cholesteryl sulphate. These salts have been shown by Gardner and Fox [1924] to behave to alkalies and acids in this manner and also to be soluble in water. We have not yet succeeded in isolating such a compound from urine though many

Table I.

Pathological condition	Cholesterol in plasma*		Urine		Cholesterol, free and in ester forms, extracted from alkaline solution by ether		Cholesterol set free after acid hydrolysis		Total cholesterol all forms mg. per 100 cc.	Cholesterol per day		Total cholesterol all forms per day mg.
	Free %	Ester %	Vol. used in cc.	No. of days	Digitonide weighed g.	Cholesterol mg. per 100 cc.	Digitonide weighed g.	Cholesterol mg. per 100 cc.		Free and ester in mg.	Hydro-lysable by acids in mg.	
Mixed normal	—	—	2000	—	—	—	—	—	0·172	—	—	1·7–2·6 †
Mixed normal	—	—	13250	—	0·0487	0·1787	0·0279	0·1024	0·2811 0·203	1·8–2·7	1·0–1·5	2·8–4·2 ‡ 2·0–3·0
Carcinoma stomach	—	—	1000	1·5	0·0049	0·119	Nil	Nil	0·119	0·8	Nil	0·8
Grave's disease	0·0476 0·0278	0·0529 0·0029	1500	2	0·0051	0·083	0·0447	0·725	0·808	0·62	5·4	6·05
Polycystic kidneys	0·0367	0·065	2540	2	0·0061	0·058	0·0021	0·0201	0·0781	0·74	0·26	1·00
Chronic nephritis	0·056	0·0912	1545	2	0·0079	0·124	0·0106	0·167	0·291	0·96	1·29	2·25
Subacute parenchymatous nephritis	0·2032 0·1374	0·4288 0·2458	3130	2	0·2329	1·81	0·1032	0·801	2·611	28·3	12·5	40·8
Subacute parenchymatous nephritis	0·212 0·0466	0·2809 0·0508	2000	1·83	0·2273	2·76	0·0832	1·01	3·77	30·14	11·03	42·4
Subacute parenchymatous nephritis	0·0993	0·6942	1400 1850 4104	2 2 5	0·0086 0·0891 —	0·149 1·17 1·128	0·1237 0·1882 0·1746	2·15 2·47 1·03	2·30 3·64 2·16	1·05 10·8 8·924	15·03 22·9 8·24	16·08 33·7 17·26 §

* These figures do not necessarily correspond exactly in date to the time of collection of the urine.
† Weight of digitonide 0·0142 g. Total all forms determined together.
‡ Assuming daily output 1·0–1·5 litres of urine.
§ Full analysis in Table II.

experiments have already been made, but this is not to be wondered at, considering the small quantities present.

It will be noticed that as far as the cases examined are concerned, the high content of ethereal sulphate, if such it be, goes hand in hand with a high plasma content of cholesterol.

Five cases of advanced renal disease are tabulated but only in the three cases of typical subacute parenchymatous nephritis which were characterised by a high plasma cholesterol content were excessively large quantities of the ethereal sulphate found. These three cases were of the type of nephritis which frequently shows the presence of large quantities of cholesterol esters in the kidney. This would suggest that in cases of marked hypercholesterolaemia, the organism tries to get rid of the excess blood cholesterol as a conjugated sulphate in the urine in the same manner as some poisonous bodies, phenols, etc.

In order to throw more light on the distribution of the cholesterol in highly albuminous urine the last-mentioned case was further examined. Four litres of urine, the output of 5 days, after careful filtration were made faintly acid with acetic acid and boiled. The urine from a previous examination had been found to contain approximately 2·6 % of protein. The precipitated protein was filtered and washed and dissolved in 2 % caustic soda. This was then thoroughly extracted with ether and the free and ester cholesterol separately determined. The free cholesterol yielded 0·0706 g. of cholesterol digitonide and the ester 0·1051 g. cholesterol digitonide. The filtrate from the proteins was then made up to 2 % content of caustic soda and repeatedly extracted with ether until no further cholesterol could be obtained. This ethereal solution by analysis in a similar way yielded 0·0112 g. digitonide from the free cholesterol and 0·0037 g. digitonide from the ester cholesterol. The thoroughly extracted urine was then evaporated to dryness, and the residue boiled with glacial acetic acid as already described and then after extraction and saponification gave 0·1746 g. cholesterol digitonide.

The percentage and daily output of cholesterol in the different fractions are set out in Table II.

Table II. *Detailed analysis showing distribution of cholesterol in an albuminous urin*

	Protein fraction		Protein free filtrate			
	Digitonide in g.	mg. per 100 cc. whole urine	Digitonide in g.	mg. per 100 cc. whole urine	Protein fraction mg. per day	Protein fr filtrate mg. per d
Free cholesterol	0·0706	0·418	0·0112	0·066	3·26	0·528
Ester cholesterol	0·0105	0·623	0·0037	0·022	4·96	0·176
Cholesterol obtained after acid hydrolysis	—	—	0·1746	1·034	—	8·24
Total, all forms	—	1·041	—	1·122	8·22	8·944

It will be noticed that 60 % of the total cholesterol, precipitated with the proteins, is in the form of ester, which is very similar to that found in plasma.

This result suggests that the bulk of the free and ester cholesterol in this nephritic urine was in combination with the proteins in much the same way as it is held in solution in plasma. It will be noticed that 91 % of the free and ester cholesterol apart from the ethereal sulphate was precipitated along with the proteins. We shall, however, have to make more experiments before we can be sure of this relationship. The free and ester cholesterol not precipitated with the proteins is of much the same order of magnitude as in normal urine but the bulk of it is in the form of free cholesterol.

The cholesterol as so-called ethereal sulphate, set free after acid hydrolysis, is present in quantities approximately equal to that found in other forms. The excretion by the kidney is obviously very minute relatively to that dealt with by the liver and scarcely affects the figures given in previous papers concerning the cholesterol balance.

Nevertheless, in considering the general cholesterol problem, this function of the kidney does not appear to be without significance.

The explanations advanced in this paper must be regarded as quite of a preliminary nature and we hope to give the results of further work in future.

SUMMARY.

Cholesterol could be determined by gravimetric estimation in all the urines examined. It exists in the urine in free and ester forms and also as a compound which is only hydrolysable by strong acids. This substance we suggest is cholesterol ethereal sulphate.

In cases of parenchymatous nephritis which were accompanied by hypercholesterolaemia there were found in the urine large increases in the content of all forms of cholesterol.

In a highly albuminous urine, the cholesterol in free and ester forms was almost wholly precipitated with the proteins on boiling with very dilute acetic acid. The precipitated cholesterol showed the same ratio of ester to total cholesterol as is found in plasma. The so-called ethereal sulphate form was still present in solution in the precipitated urine in approximately the same quantity as the total other forms in the whole urine.

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CI. STUDIES ON XANTHINE OXIDASE. VI.

A CELL OXIDATION SYSTEM INDEPENDENT OF IRON.

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Warburg [1921; 1923, 1, 2] has developed a theory of oxidations in the cell which states that molecular oxygen must always be activated by iron before it can oxidise organic substances. He considers iron to be the only substance in the cell with which molecular oxygen reacts: "Das Oxydationsmittel der Atmung ist der molekulare Sauerstoff, die Primärreaktion der Atmung die Reaktion zwischen molekularem Sauerstoff und Eisen, und *nur* in dieser Reaktion, nicht mit den organischen Molekülen, vermag der molekulare Sauerstoff in der Zelle zu reagieren."

His theory is based, partly on the study of the respiration of the sea urchin's eggs, partly on the consideration of certain chemical systems, namely, the oxidations occurring on the surface of iron-containing charcoal, the oxidation of sulphydryl compounds, and the oxidation of fructose in the presence of phosphate. In all these systems the addition of cyanide inhibits the oxidation, and the addition of minute quantities of iron markedly catalyses the reaction.

Although Warburg extends his theory to include all oxidations occurring in the cell, the only evidence with regard to enzyme systems is afforded by the work of Fleisch [1924] and Szent-Györgyi [1924] on the succinic oxidase of animal tissues. These workers found that the addition of cyanide inhibited the oxygen uptake by succinate in the presence of the enzyme, but had no effect upon the reduction of methylene blue by the same system. They concluded that in this and similar systems oxygen activation was necessary, and that the cyanide inhibited the activator. These experiments would seem to show that Warburg's view of oxygen activation by iron applies also to enzyme systems.

Experimental evidence will, however, be given in this paper to show that this is not the case with the xanthine oxidase system. Moreover, we have recently obtained results, which will be published later, showing that in the succinic oxidase system it is very doubtful whether oxygen activation by iron occurs.

A detailed study of the xanthine oxidase of animal tissues has been made by the authors [1924, 1, 2, 3; 1925] and, as this enzyme can easily be prepared free from other enzymes, it was thought to be of interest to see whether oxygen activation by iron was necessary in this system. The experiments were carried out by means of the Barcroft respirometer, the technique of which has been previously described. In all cases identical solutions were placed in both flasks of the apparatus, but hypoxanthine, the oxidisable substance, was placed only in one flask. This ensures that the experiments are properly controlled. All experiments were carried out at 40° and at p_H 7·6.

We have already shown [1924, 2] that the reduction of methylene blue by the xanthine oxidase system is practically unaffected by cyanide, except in concentrations exceeding $M/100$, when a slight inhibition is produced. This is due to a slight destruction of the enzyme itself. We have found the oxygen uptake of the system to be affected by cyanide in exactly the same way as was the reduction of methylene blue. Cyanide in concentrations as high as $M/100$ produced no inhibition in the rate of oxygen uptake, whereas, in the systems previously mentioned, other workers have found concentrations of the order of $M/1000$ to produce complete or almost complete inhibition.

The following typical experiment shows that the xanthine oxidase system is not inhibited by cyanide.

The contents of the flasks were:

A. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 1·5 cc. p_H 7·6 phosphate buffer.

B. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 0·1 cc. $M/10$ KCN + 1·4 cc. p_H 7·6 phosphate buffer.

C. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 0·2 cc. $M/10$ KCN + 1·3 cc. p_H 7·6 phosphate buffer.

D. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 0·5 cc. $M/10$ KCN + 1·0 cc. p_H 7·6 phosphate buffer.

All solutions were adjusted to p_H 7·6 before addition. The xanthine oxidase was prepared from milk by the method described by us [1924, 1].

Since the effect of cyanide is negative, the curves are all of the same form and hence only isolated points are quoted. The figures denote oxygen absorbed in cubic millimetres corrected to normal temperature and pressure and to dryness.

Time in minutes	*A* (no KCN)	*B* (KCN = $M/300$)	*C* (KCN = $M/150$)	*D* (KCN = $M/60$)
20	60	72	63	55
55	124	138	110	100
80	143	145	126	103

It can be seen that even relatively high concentrations of cyanide have practically no effect on the oxygen uptake. The uptake in *D* is, however, somewhat smaller than in the others. This was shown to be due to destruction of the enzyme by the cyanide, by testing the solutions at the end of the experiment with methylene blue and an added trace of hypoxanthine. Practically no enzyme was left in either flask of *D*, whereas in *A*, *B* and *C* there still remained a considerable quantity of active enzyme. This destruction in *D* must not be confused with the destruction of the enzyme by the hydrogen peroxide normally formed in the reaction, which occurs in the experimental flask only

[Dixon, 1925]. No peroxide is formed in the control flask, so that the destruction observed here must be due to the cyanide.

In order to eliminate any possibility that the xanthine oxidase in milk might behave differently from that in the tissues, an experiment was carried out on a liver suspension which contained much xanthine oxidase, and it was found that in this case also cyanide produced absolutely no inhibition of the oxygen uptake by this oxidase system, although in the same preparation a considerably smaller concentration of cyanide completely inhibited the oxygen uptake of the succinic oxidase system. It is clear that unless a specific oxygen activator is required for each enzyme, an assumption highly improbable, the xanthine oxidase system does not require active oxygen. The inhibition of the succinic system is due, according to Warburg's theory, to the prevention of oxygen activation, but, although no active oxygen is present, the xanthine oxidation still occurs. It is therefore clear that the xanthine oxidase system, since it is not affected by cyanide, forms an exception to the general statement made by Warburg that cyanide inhibits all tissue respiration systems.

Warburg and Sakuma [1923], Harrison [1924] and Meyerhof and Matsuoka [1924] have shown that pyrophosphates can replace cyanide in the inhibition of iron catalysis. We have repeated the preceding experiment on the xanthine oxidase, using pyrophosphate instead of cyanide, and again have obtained no inhibition. The maximum concentration of pyrophosphate used by us was $M/20$, whereas Harrison found that the oxidation of glutathione was markedly inhibited by $M/50$ pyrophosphate.

These two experiments afford strong evidence that iron plays no part in this system, since the concentration of inhibiting substance was far larger than that required to produce inhibition of iron catalysis.

Further evidence is afforded by observations made on the effect of the addition of iron to the xanthine oxidase system. Warburg, Harrison and Meyerhof and Matsuoka have found that, in all the systems which they have investigated, the addition of very small amounts of iron salts produces a marked acceleration of the oxygen uptake. We therefore added iron in varying concentrations to the xanthine oxidase system. The following experiment shows that no acceleration was produced even by relatively high concentrations of iron.

The contents of the flasks were:

A. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 1·5 cc. p_H 7·6 buffer.

B. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 0·1 cc. $FeCl_3$ (0·1 mg. Fe) + 1·4 cc. p_H 7·6 buffer.

C. 1 cc. xanthine oxidase solution + 0·5 cc. (1 mg.) hypoxanthine + 0·5 cc. $FeCl_3$ (0·5 mg. Fe) + 1·0 cc. p_H 7·6 buffer.

The figures again represent cubic millimetres of oxygen absorbed.

Time in minutes	*A* (no Fe)	*B* (0·1 mg. Fe)	*C* (0·5 mg. Fe)
10	40	36	37
20	69	65	66
55	115	104	112

The ferric chloride solution was not neutralised, but it was found that the addition of the amount in question produced no appreciable change in the p_H of the solution. It is not possible to add an amount of iron greater than that in *C* when using phosphate buffer, as the limit of solubility of ferric phosphate is here reached.

The figures show that no acceleration of the oxygen uptake of the xanthine oxidase system is produced by the addition of an iron salt.

We have, then, in the xanthine oxidase a system occurring in the cell in which molecular oxygen brings about oxidation without having been activated by iron. Warburg's statement quoted above that molecular oxygen can react only with iron, and not with organic molecules, is thus shown to be incorrect. Molecular oxygen seems to act as a direct acceptor of hydrogen in this system, as it has been shown [Thurlow, 1925] that hydrogen peroxide is formed as an intermediary product of the reaction. The system therefore forms an exact illustration of Wieland's [1922] theory of oxidation.

SUMMARY.

1. The oxidation of hypoxanthine by molecular oxygen in presence of the xanthine oxidase is not inhibited by cyanide or pyrophosphate.

2. The addition of iron to the system produces no acceleration.

3. Oxygen activation by iron does not occur in this system, which thus forms an exception to Warburg's statement that molecular oxygen can react only with iron and not with organic molecules.

We wish to express our thanks to Sir F. G. Hopkins for his interest in this work. One of us (M. D.) is also indebted to the Royal Commissioners for the Exhibition of 1851 for a senior Studentship.

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CII. THE PECTIC SUBSTANCES OF PLANTS. PART III.

THE NATURE OF PECTINOGEN AND ITS RELATION TO PECTIC ACID.

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In previous communications [Schryver and Haynes, 1916; Clayson, Norris and Schryver, 1921] the preparation and properties of pectinogen and cytopectic acid were described, and the results obtained indicated that the latter was a definite chemical compound. The object of the present communication is to establish more definitely the nature of pectinogen as it occurs in the plant and on extraction in the laboratory, and its relation to cytopectic acid and the other constituents of the cell-walls of plants.

The pectic substances have been the subject of researches undertaken by a number of investigators, with the unfortunate result that some confusion in nomenclature has arisen. The term pectin has been used to describe every conceivable type of pectic substance. Pectinogen has also been widely used to describe a substance different from pectin and usually considered to be the precursor of pectin. As such it has also been called protopectin [Tutin, 1923, 2] and recently the old term pectose has been revived [Carré, 1925].

Now, there is very little doubt that the basal molecule of the pectic substances is that referred to above, cytopectic acid, and this name was introduced by us in an attempt to arrive at some standard nomenclature; there is also little doubt that this substance exists in a modified form in the cell-wall, and this precursor we called pectinogen. This distinction has been adopted in effect by recent workers [Nanji, Paton and Ling, 1925], who refer to pectic acid and its modification, pectinogen. We shall therefore adhere to this system in order to avoid further confusion.

Very little is known of the nature of pectinogen *in situ* and the contradictory and controversial statements that have been made arise in some measure from the lack of any standard of nomenclature, preparation and analysis.

Before dealing with pectinogen itself, it is necessary to consider briefly the nature of the basal molecule, pectic acid. A considerable advance in our knowledge of the constitution of this substance has been made recently. Ehrlich [1917] showed that the molecule contained galacturonic acid groups together with arabinose and galactose. These products of hydrolysis have been obtained in this laboratory, using a modification of a method due to Monier-Williams [1921]. The paper by Nanji, Paton and Ling contains a further important contribution to our knowledge of pectic acid. The carbon dioxide evolved on hydrolysis was quantitatively determined and from the results obtained it became apparent that the molecule must consist of four molecules of galacturonic acid with one molecule each of arabinose and galactose; they consider these molecules to be combined in a six-membered ring each side corresponding to the appropriate carbohydrate or acid molecule, the carboxyl groups of the acid being left free (see p. 685).

The empirical formula of this acid is $C_{35}H_{50}O_{33}$ and this agrees very well with the analytical results carried out both by them and by us on pectic acid; the formula tentatively suggested by Schryver and Haynes [1916], $C_{17}H_{24}O_{16}$, is a close approximation to the above. This was proposed as a formula for pectinogen, however, and in view of the fact that it has been used in connection with a method for estimating pectic acid, it is important that the corrected formula be speedily adopted.

Any attempt to correlate pectic acid and pectinogen should have reference to the above formula and it will be shown later that this formula helps to explain some of the problems which have arisen in connection with pectinogen. In the early paper of Schryver and Haynes [1916] it was suggested that the action of alkalies effected the change from pectinogen to pectic acid, and von Fellenberg [1918] showed that this change also involved the liberation of methyl alcohol. The first statement has been universally accepted, although perhaps on somewhat slender evidence, but the second has suffered some amplification and criticism. Tutin [1923, 1] states that he obtained from apple "pectin" both methyl alcohol and acetone in sufficient quantity to enable him to determine the physical constants and chemical nature of these substances. The presence of acetone in addition to methyl alcohol has been refuted by several workers and we, also, regard methyl alcohol as the sole volatile product of the reaction. This is founded on analytical methods it is true, but two methods of estimation were used, that of Zeisel and another due to Schryver and Wood [1920], the latter so delicate that the presence of acetone would be bound to affect the results to a considerable extent, whereas in fact they agreed very closely by the two methods. The previous history of Tutin's "pectin"—the apples were taken from a cider-press—would surely account for the presence of such volatile substances as impurities and not as definite groups in the molecule of the pectic substance.

Nanji, Paton and Ling [1925] agree with us in regarding the action as one of de-esterification and reiterate our statement that the analyses do not warrant the assumption that any methylpentoses exist in the molecule. We, however, while regarding the main reaction as one of de-esterification, have found that there is also another factor to be considered. In addition to methyl alcohol, we have found another substance, which is non-volatile, in the alkaline extract, and this substance appears to belong to the class described as hemicelluloses. It appears to be associated with the main bulk of pectic substance, whether as a mixture or in loose combination is not as yet quite certain.

Some indications of the nature of pectinogen as it exists *in situ* were obtained and it seems probable that the formula for the basal molecule adapts itself very well to our results. Thus we regard pectinogen *in situ* as consisting of a methylated pectic acid in which three of the carboxyl groups are esterified, thus leaving one still free to account for the definitely acidic nature of pectinogen, in loose combination or admixture with a substance similar to the hemicelluloses.

The positions of the linkages in the molecule are as yet unknown but we have some evidence that they are not all of the same type. We consider that there is an intermediate product in the process of de-esterification containing only one methoxyl group, which seems to be more stable than the more completely methylated compound and consequently less susceptible to the action of weak alkalies.

We may now consider in more detail the basis of the above statements.

Preparation of pectinogen and its properties.

At the outset, the methods adopted for the extraction of pectinogen were similar to those used in the case of cytopectic acid, with the difference that no extraction with caustic soda took place before the pectic substance was obtained. The maximum yield was the prime consideration, and to this end the plant tissues were extracted to exhaustion and the fractions combined and precipitated as one. A number of extractives were used and after a time the large discrepancies in analytical results made the fact apparent that pectinogen was not of such constant composition as was cytopectic acid. It became obvious that the method of exhaustive extraction must be abandoned and that the pectinogen should be prepared under rigidly standardised conditions, having due regard to the factors involved. The most important of these factors were found to be:

(i) Nature of source.
(ii) Nature of extractive.
(iii) Concentration of extractive.
(iv) Time of extraction.
(v) Temperature of extraction.

Each of these factors had a varying effect on the pectinogen in the following respects:

(i) Yield.
(ii) Purity. (Freedom or otherwise from adsorbed inorganic salts.)

(iii) Yield of furfural on analysis.

(iv) Yield of methyl alcohol on analysis.

Before proceeding to the examination of the effect of the above factors the experimental method of exhaustive extraction and the results obtained may be given.

The chief sources employed were turnips, onions and, to a lesser extent, pea-pods. The method of preparation of the cell-wall substance was that of Clayson, Norris and Schryver [1921]. The yield is small and, consequently, large quantities of raw material have been dealt with, amounting to about half a ton each of turnips and onions. Typical yields of cell-wall substance are, for turnip, 4 % and for onion, 2·25 % of the raw material.

In general, the air-dried cell-wall substance was treated with twenty times its weight of extracting solution and kept at about 90° in a water-bath for some time. Filtration followed and the residues were again extracted with an additional quantity of solution; this was repeated until the filtered extracts contained no pectinogen. Exhaustion was usually reached at the fifth extraction. The united extracts were then poured into two volumes of alcohol and after standing overnight the resulting gel was filtered through ordinary funnels. The gel is soluble in water and for this reason was difficult to precipitate in cases where the concentration of pectinogen in the extract was small, additional alcohol sometimes being required before the slimy mass was sufficiently coagulated to filter easily; in all cases the filtration was slow until the gel was placed in higher grades of alcohol. After allowing the product to stand in graded strengths of alcohol, the final drying was effected by absolute alcohol and ether, and air-drying for some days by means of a fan. Previous to analysis the pectinogen was dried over phosphorus pentoxide in a vacuum desiccator at room temperature. At this temperature constant weight was usually attained after a fortnight; this slow drying, however, was necessary, as the use of a vacuum dryer at 100° was found to be detrimental to the product, charring frequently resulting.

Pectinogen prepared by this method was found to be white and amorphous; it is brittle and impossible to powder by hand although susceptible to mechanical grinding. On standing with water, it swells considerably during the period of imbibition and finally goes into solution; the process is considerably accelerated by mechanical stirring. For general purposes a 1 % solution was found to be the maximum for easy working as the viscosity becomes high and solution slow and difficult.

Pectinogen contains methoxyl groups as shown by the action of hydriodic acid (method of Zeisel); on distillation with 12 % hydrochloric acid, furfural is obtained indicating the presence of pentose groups and/or sugar acids of the glycuronic acid type; when carefully oxidised with nitric acid mucic acid is obtained, indicating the presence of galactose or galactans.

Pectinogen appears to exist in the cell-wall in combination with calcium, and it has been suggested that magnesium may also be present in some form. The evidence that calcium is in combination with the pectic complex is very strong, since it invariably appears that pectinogen is extracted by agents which throw down an insoluble calcium salt by double decomposition, leaving the pectinogen in solution. Ammonium oxalate and oxalic acid are familiar examples and even carbon dioxide is capable of liberating appreciable quantities of pectinogen. On the other hand, salts such as sodium chloride are unable to effect the change.

Nanji, Paton and Ling [1925] lay some stress on the presence of iron in the ash obtained from pectinogen. This has been noticed by Carré and Haynes and also by ourselves; no quantitative work has been carried out, however, and we do not consider that there is sufficient evidence at present to justify the assumption that iron is an essential constituent, although this is one of several interesting possibilities.

Note on the analytical methods employed. The principal determinations carried out on each sample of pectinogen were: ash content, yield of furfural, methoxyl content by two methods. As it is now firmly established that the basal molecule contains galacturonic acid, we have abandoned the practice of calculating furfural yields as pentose and give actual yields of furfural obtained by Tollens' method. Methoxyl groups were estimated by the well-known method of Zeisel and also by that of Schryver and Wood [1920] and are dealt with more fully later.

In some cases the yield of mucic acid was estimated, the results being calculated as galactose or galactan, and adsorbed salts were estimated where possible. Results quoted throughout are the averages of at least two determinations, concordant within the limits of experimental error, and are calculated on an ash- and salt-free basis.

Influence of source on pectinogen.

In the cases now to be considered, the method of extraction has been described and it is important to remember that the final product was the maximum amount of pectic substance that could be extracted, and that if the product is unstable under the conditions of extraction, variations in composition as shown by analysis are to be expected.

Yield of pectinogen from various sources.

Raw material	Weight of cell-wall substance taken in g.	Weight of pectinogen in g.	Yield %
Turnips	100	24	24
	200	34	17
	100	20	20
	250	54	21·6
Onions	25	4	16
	100	17	17
	150	22·5	15
	150	24	16
Pea-pods	100	8	8

In the above experiments, ammonium oxalate was used as the extractive and the yields given are the total weights of pectinogen obtained after repeated extraction. It will be seen that turnips give the best yield although for some purposes to be detailed later onions are more suitable, in spite of the slightly smaller yield.

The effect of the source on pectinogen as shown by analytical results is indicated below. The extractives used are ammonium oxalate and 0·5 % oxalic acid, the cell-wall substance being exhaustively extracted at a temperature of 85–95°.

Source	Extractive	Ash %	Contaminating salts %	Furfural %	Methoxyl %
Turnips	Am. oxalate	3·12	15·7	20·32	7·1
	Oxalic acid	2·56	—	19·35	9·2
Onions	Am. oxalate	5·65	5·15	13·95	2·1
	Oxalic acid	2·44	—	9·06	2·2
Pea-pods	,,	3·34	—	9·21	1·9

It will be seen from the above results that there is no uniformity in the product obtained, the furfural yields varying between 20 % and 9 % and the methoxyl content between 9·2 and 1·9 %. The only concordance to be observed is in the samples extracted from onions and pea-pods by oxalic acid, but these are in violent contrast with the others. The variability in composition of the product is thus clearly demonstrated and may be attributed to a variety of causes. The fact that each source used is at a different stage of growth may account for variations in the products to some extent, and it is certain that the action of the extractive agent is responsible for a change in the composition of the resulting product.

Another important point, bearing on the technique of extraction, arises from the foregoing results in that it will be noticed that two estimations of adsorbed salts are given. The retention of varying quantities of the substance used for extraction was to be expected, but was nevertheless a source of some trouble. The ordinary ash determination did not indicate the presence of ammonium oxalate, for instance, and it was necessary to estimate the nitrogen in the sample by the method of Kjeldahl in order to arrive at the correct value of the inorganic constituents. In some cases it was very difficult to estimate these contaminating salts as the gel itself prevented the use of the usual methods.

Apart from the difficulties of analysis, it was necessary to obtain the products in as pure a state as possible and methods of eliminating impurities were sought.

The method finally adopted was one of repeated solution in water and reprecipitation with alcohol. After three precipitations in this manner, over 90 % of the inorganic impurities were completely removed, and solutions were obtained from which it was difficult, but not impossible, to precipitate pectinogen by means of alcohol.

Dialysis followed by reprecipitation with alcohol was found to be unsatisfactory, since after dialysis precipitation with alcohol could only be effected by adding either a salt, which reintroduced ash, or an acid, which affected the constitution of the gel.

Effect of extractive on pectinogen.

In order that the results under this heading should be more strictly comparable, one source only was used, all the cell-wall substance being derived from the same batch of turnips.

In each case the pectinogen was produced by exhaustive extraction with 0·5 % solutions, except in the last case quoted. In this the residues were placed in twenty volumes of water maintained at 90–95°, carbon dioxide being passed into the stirred mass for 6–8 hours. The analytical results are as follows:

Extractive	Yield %	Furfural %	Methoxyl %
Ammonium oxalate	20	20·32	7·1
Oxalic acid	20	19·35	9·2
Ammonium sulphate	10	23·16	7·4
Carbon dioxide	2·5	19·09	4·7

The furfural yield for the sample prepared with ammonium sulphate is quite abnormal and the chief effect of the extractive is observable in the yields, which show that ammonium oxalate and oxalic acid are the most effective.

Another result of some significance is noticeable in the case of the methoxyl content in the first two extractives above. It is known that the action of alkalies on pectinogen causes the elimination of methyl alcohol, and the lower methoxyl value in the first case above is due to the prolonged action of ammonium oxalate, which is slightly alkaline at the temperature and dilution of the extraction. One of the variants in the composition of pectinogen is thus seen to be a loosely combined methoxyl group or groups.

Other factors affecting the nature of pectinogen.

The effect of the temperature of extraction was found to be slight although the yields obtained at lower temperatures were much smaller. Cold reagents did not attack the cell-wall substance at all; after extraction of 100 g. of cell-wall substance for several hours at 36–37°, a small quantity of pectinogen was obtained which was just sufficient for analytical purposes.

In the table below, the source was onions and the extractive was 0·5 % oxalic acid:

Temperature of extraction	Ash %	Furfural %	Methoxyl %
37·5°	2·73	9·06	3·4
80·0°	2·44	9·06	2·2

The effect of temperature on the furfural yield was negligible but there is again a change in the methoxyl value under more drastic conditions of extraction, giving further evidence of the inherent instability of this group.

This fact was further exemplified in another experiment, in which an ammonium oxalate extract containing pectinogen was heated at 100° under a reflux condenser for 8 hours before precipitation. Thus the pectinogen was in intimate contact with the extractive during this period. A comparison of analyses of a portion (*a*) freshly precipitated, and (*b*) after the above treatment, shows again the loss in methoxyl groups due to the prolonged action of the extractive:

Sample	Furfural %	Methoxyl %
(*a*)	20·32	7·1
(*b*)	20·64	5·0

The yield of mucic acid from pectinogen.

In view of the fact that the basal molecule of pectic acid and pectinogen contains one molecule of galactose and four molecules of galacturonic acid or its methylated analogue, we might expect that large yields of mucic acid would be obtained on oxidation with dilute nitric acid. This was found to be the case although the actual yields obtained were never those indicated by theory. The acid was estimated by the method of Tollens in which a weighed quantity of the product is gently evaporated with nitric acid of sp.G. 1·15 until the reaction is complete and a clear solution is obtained. The precipitate which forms on standing is re-dissolved in ammoniacal ammonium carbonate and slowly evaporated to dryness. The mucic acid is finally precipitated by addition of more nitric acid and weighed after filtration and drying in a Gooch crucible.

Nanji, Paton and Ling [1925], seem to have experienced some difficulty with this estimation, but we have obtained yields of mucic acid from pectinogens corresponding to 40–50 % galactose, and from pectic acid amounts equivalent to 50–60 %. We have found that the method required very careful working and that it was necessary to evaporate very slowly for 2 days or more in order to ensure maximum yields.

The yields obtained show considerable variations, due in part to the varying nature of the pectinogen and also, doubtless, to the estimation itself. They are considerably higher than those found by the above workers, but even now are not as high as theory requires. We have detected oxalic acid in appreciable quantity in the mother liquor after filtering off the mucic acid.

The table shows the yields of mucic acid calculated as galactose from pectinogen, the last three estimations referring to pectic acid.

Source	Method of extraction	Galactose %
Turnips	0·5 % oxalic acid at 90°	40·9
Onions	0·5 % oxalic acid at 90°	34·9, 37·0, 49·5
Onions	0·5 % ammonium oxalate at 90°	50·9
Onions	Pectic acid	50·1, 64·0
Turnips	Pectic acid	58·7

The above figures are the averages of several estimations, each one referring to a different sample.

In the experiments which follow, pectinogen was extracted under more standardised conditions and the effect of the concentration of the extractive and the time of extraction more fully examined.

Effect of concentration of extractive.

In this series of experiments turnip residues were used which, after the usual washing, pressing and drying, had been finely ground and passed through a fine sieve in order to ensure uniformity as far as possible. Ammonium oxalate was used as the extracting agent and in strengths of 1 %, 0·5 %, 0·25 %, 0·1 % and 0·05 %.

In each case, 20 g. of residues were weighed out and placed in a conical flask, 200 cc. of water were added, and, after about 2 hours, 200 cc. of ammonium oxalate solution. The mixture was heated in a water-bath under reflux for exactly 1 hour, then rapidly cooled, filtered through muslin and paper-pulp, and the pectinogen precipitated and dried as usual.

It was found that the best yield was obtained in the experiments where 0·5 % and 0·25 % oxalate had been used; nothing was gained by increasing the concentration to 1 %, and the extracts after treatment with 0·1 % and 0·05 % oxalate contained such small quantities of pectinogen that precipitation was impossible.

The variation in concentration of the extractive had little effect on the nature of the final product.

The following gives the results in tabular form:

Concentration of extractive %	Yield (approx.) %	Ash %	Furfural %
1·0	10	1·50	18·43
0·5	13	1·72	18·69
0·25	12	1·39	18·60
0·1	—	—	—
0·05	—	—	—

It was decided that for general purposes no advantage was to be gained by departing from the usual practice of using 0·5 % solutions.

Effect of time of heating on pectinogen.

In these small scale experiments a standard method was employed and the pectinogen produced was the result of a single extraction, no additional heating or washing taking place after the period of extraction decided on. The pectinogen was usually redissolved and reprecipitated to eliminate the greater part of the inorganic salts adsorbed in the first precipitate.

The procedure was as follows: 50 g. of air-dried residues were allowed to stand for 1 hour with 500 cc. of 0·5 % ammonium oxalate solution, after which a further 250 cc. were added and the mixture, in a flask under reflux, heated in a water-bath to 90–95° for a definite period. At the end of a stated time the mixture was rapidly filtered through muslin and paper-pulp in a Buchner funnel and flask. The extract was poured into two volumes of 95 % alcohol, filtered, washed, redissolved, and reprecipitated. After the usual treatment it was dried to constant weight in a vacuum desiccator over phosphorus pentoxide.

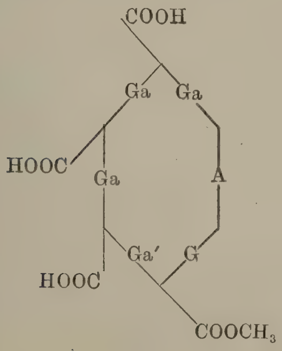
In a series of experiments under the conditions indicated, using turnips as the source of the pectinogen, the following results were obtained:

Time of heating in hours	Ash %	Furfural %	Methoxyl %
0·5	1·6	18·48	8·21
2·0	1·2	18·86	6·61
4·0	0·96	19·13	5·89
24·0	1·95	19·45	3·19

A short preliminary report to the Food Investigation Board for 1923, published in 1924, embodies some of these results in a modified form.

Interesting points arise from these results, and from them it is possible to form some idea of the nature of pectinogen as it exists in the cell-wall. By extrapolating the curves to zero time we have a hypothetical pectinogen containing 9·1 % methoxyl and yielding 18·27 % furfural. Now, a compound having the formula shown below, but in which three carboxyl groups of the basal molecule are methylated, should yield 8·94 % of methoxyl and 19·17 % furfural. The latter figure is only an approximation, being based on a yield of furfural from the pentose group equal to 90 % of the theoretical value and from the galacturonic acid groups of 31 %, these being generally accepted figures. We did not anticipate complete agreement with theory owing to the suspected presence of another substance in small quantity in association with the main product.

Another point arises in the consideration of these curves. It will be noticed that the results are almost constant at periods corresponding to 18 hours and longer. There is thus evidence that the product formed at this period is somewhat more stable under the slightly alkaline conditions of the reaction. The product obtained after extracting for 24 hours contains 3·19 % methoxyl and yields 19·45 % furfural. A substance having the formula shown herewith, in which the basal molecule contains only one methylated carboxyl group, gives theoretical yields of 3·05 % and 20·3 % of methoxyl and furfural respectively, calculated as above. There is thus the probability that this compound is the main product at this stage of the extraction, although again differences from theory may be expected due to the presence of another substance. It will be remembered that 4 % sodium hydroxide is generally used to effect the complete change to pectic acid, and it is probable that this



G = Galactose

A = Arabinose

Ga = Galacturonic acid

Ga′ = Methylated galacturonic acid

compound is only completely de-esterified by the action of more strongly alkaline solutions than the hot ammonium oxalate; the positions of the various linkages in the molecule, at present unknown, may explain the comparative stability of this substance.

The methoxyl content of pectinogen.

It has been stated previously that methyl alcohol is one of the products of the action of alkalies on pectinogen. The preceding experiments show that the amount liberated varies according to the source of the pectinogen, the nature of the extractive, and the method and time of extraction.

In addition, the question has arisen as to whether the methyl alcohol thus obtained is the only product of the reaction. Tutin [1923, 1] states that acetone in addition to methyl alcohol was found in pectin from apples after use in a cider-press. In the case of pectinogen prepared by the usual methods we have been unable to find any evidence of the existence of acetone (see p. 677).

It is advisable to give the methods adopted in some detail in order that the evidence for the above statements may be more easily followed. The method of Zeisel was followed with but slight modifications now in common use. The products under examination were dried to constant weight over phosphorus pentoxide in a vacuum desiccator, more than a fortnight sometimes being required to effect this at room temperature. An accurately weighed sample in a capsule was placed in the Zeisel flask with 10 cc. of hydriodic acid of sp.g. 1·7. Carbon dioxide was passed into the reaction flask, which was connected to two flasks containing alcoholic silver nitrate solution; the reaction was carried out at 130–135°, methyl iodide being swept into the receiving flasks and silver iodide consequently precipitated. This was washed, dried and weighed in the usual manner.

The method proved of general utility in dealing with these products and gave results at least as accurate as those obtained in more orthodox organic chemical practice, as the following list shows. Duplicate results of estimations on different samples are shown:

(i) 3·77, 3·80. (iii) 6·74, 6·80.
(ii) 3·18, 3·27. (iv) 8·30, 8·37.

The method of Schryver and Wood [1920] is a micro-method of great delicacy, depending on the oxidation of methyl alcohol to formaldehyde by ammonium persulphate. The reaction is followed colorimetrically, by means of the well-known colour reaction of formaldehyde with phenylhydrazine and potassium ferricyanide. The great accuracy of the method is the chief recommendation for the process, which takes considerably longer to accomplish than the older method of Zeisel. In estimating the methyl alcohol liberated from pectinogen, a standard solution of the sample was prepared and accurate aliquot parts placed in conical flasks. An equal quantity of 8 % sodium hydroxide was added and the mixture allowed to stand with occasional shaking for 1 hour. Any gel that had formed was filtered off and a definite quantity of the filtrate containing methyl alcohol was run into a distilling flask; the liquid was neutralised with glacial phosphoric acid and distilled into a receiver surrounded with a freezing mixture. An elaborate system of flasks acting as traps was also maintained at about – 5° and distillation was carried out at such a rate that the drops froze in the first receiver as they came over. On melting, the distillate was made up to definite volume and estimated in the above manner.

It is obvious that the two methods are totally different in principle and procedure and it is of importance from the present point of view to note that the second method is very susceptible to the presence of ethyl alcohol and acetone, these two substances reacting under the conditions of experiment and thus causing big differences in the results. It would thus be legitimate to assume that if concordant results, within the limits of experimental error, are obtained by the two methods, then the only volatile product of the action of the alkali on pectinogen is methyl alcohol. The table gives a comparison of results by the two methods. They agree so well that the first method was adopted as the standard method, since it is quicker, the second being used in special cases.

	Methoxyl %	
Sample No.	Zeisel	Schryver and Wood
1	7·1	6·9
2	2·1	2·2
3	2·2	2·3
4	1·9	1·8
5	7·4	7·3
6	3·4	3·2
7	2·3	1·9
8	7·1	7·2

The conversion of pectinogen into pectic acid.

It has been stated and generally accepted that the main product of the action of alkalies on pectinogen is pectic acid. The object of the experiments now to be described was to determine the most suitable alkali for the purpose, and to discover whether this substance was the sole non-volatile product of the reaction. If there is another product of the reaction it must exist as a soluble substance in the alkaline extract after removal of the insoluble pectic acid. The choice of an alkali must therefore be governed by this consideration, and it would seem advisable to use one which can be easily eliminated from solution. It seemed feasible to employ lime or baryta water, thus effecting the alkaline change and the precipitation of a gel in one operation. It was not known, however, whether these alkalies were sufficiently strong to effect the change, and in order to show that the main insoluble product of the reaction was pectic acid the following procedure was adopted. A solution of pectinogen, about 1 % strength, was allowed to stand with an equal volume of saturated lime water in one case, and saturated baryta water in the other, for a period of 24 hours. The gel, which formed immediately, was thoroughly washed with water and then allowed to stand in moderately concentrated hydrochloric acid, several changes being given. By this means the calcium or barium gel was converted to pectic acid and the inorganic constituents eliminated almost entirely. Further washing took place until the washings no longer reacted acid, and then the gel was dried in graded strengths of alcohol as usual. The products were vacuum-dried at room temperature to constant weight before analysis.

The products obtained all contained less than 1 % ash, showing that we were dealing with the acid and not the salt. The yield of furfural, calculated on an ash-free basis, on four products obtained by the action of lime water was: 21·42, 22·33, 22·39, 22·12; average 22·06 %. The product of the action of baryta gave as an average of two determinations, 21·94 %. These figures are within very close limits of those usually obtained for pectic acid, and we may reasonably conclude that these alkalies are effective.

Owing to the ease with which these bases can be removed from solution they were of great use in the examination for soluble products of the reaction on a larger scale. Evidence of the existence of such products was obtained in a somewhat unusual manner.

Anomalies in the methyl alcohol content of pectinogen.

The series of experiments undertaken at this point was designed to determine the rate at which the methyl alcohol was liberated from pectinogen solutions by the action of lime-water.

The pectinogen used in this series was prepared by heating the residues with 0·5 % ammonium oxalate solution for 24 hours, and gave the following analyses:

Ash: 1·95 %; Furfural: 19·45 %; Methyl alcohol: 3·29 % (Zeisel).

A standard solution of pectinogen (about 0·6 %) was prepared and 50 cc. placed in each of five conical flasks; 50 cc. of saturated lime-water were then added and the flasks, loosely stoppered, allowed to stand in the ice-chest for 3, 5, 24 and 72 hours. At the end of the allotted time, the mixture was centrifuged, the calcium gel being thrown down, and 25 cc. of the supernatant liquid were made up accurately to 100 cc. The methyl alcohol in this solution which contained the equivalent of 0·053 g. pectinogen per 100 cc. was determined by Schryver and Wood's method without distillation. The following are the results obtained:

Sample No.	Period of reaction hrs.	Critical concentration of persulphate %	CH_3OH %	Pectinogen %	CH_3OH 100 g. pectinogen	Difference
1	1·3	0·143	0·0084	0·053	11·56	**8·27**
2	3·3	0·153	0·0090	—	12·39	**9·10**
3	5·3	0·174	0·0102	—	14·04	**10·75**
4	24·0	0·266	0·0157	—	21·48	**18·19**
5	72·0	0·255	0·0151	—	18·50	**15·11**

The figures in thick type in the final column indicate the difference between the observed apparent methyl alcohol content and the value obtained by the method of Zeisel. The results indicate (i) that about 24 hours is the optimum time for the reaction; (ii) that there must be some substance other than methyl alcohol present in the alkaline extract. This substance must be capable of reducing the ammonium persulphate used in the above method, thus giving a falsely high apparent methyl alcohol content. As will be shown later, large scale experiments resulted in the isolation of this substance. Thus we have as products of the action of alkalies on pectinogen: pectic acid, methyl alcohol and a new substance, shown later to have many properties in common with hemicelluloses previously described.

Isolation of a hemicellulose from pectinogen.

1. *Preparation of pectinogen.* 400 g. of the dried cell-wall substance of turnips were allowed to stand with 4000 cc. of water for about 2 hours, when all the water had been taken up. 4000 cc. of 1 % ammonium oxalate solution were then added with constant stirring. The mixture was placed in large flasks and the temperature maintained at 90–95° for 24 hours by means of a constant-level water-bath.

On cooling and filtering the extract amounted to about 6 litres and the pectinogen was precipitated by pouring this into about 10 litres of 95 % alcohol. The precipitate was very gelatinous and filtered slowly, and when most of the alcoholic liquid had passed the filter, the gel was redissolved in water and again precipitated. The pectinogen was finally dried by standing in graded strengths of alcohol and ether, and then in a current of air from a fan. The air-dry weight was 68 g., representing 17 % of the residues.

2. *Separation of the hemicellulose.* 65 g. of the air-dried pectinogen were dissolved in 6500 cc. of distilled water, and an equal quantity of saturated lime-water added with stirring. The mixture was allowed to stand for 24 hours, and then the gel was filtered off and washed thoroughly. The filtrate and washings were saturated with carbon dioxide to remove excess of lime, and again filtered. The final volume of the liquid, after concentration *in vacuo*, was about 100 cc. and this was filtered into 300 cc. of alcohol. A light flocculent precipitate came down gradually and settled on shaking. This was dried in a vacuum desiccator over phosphorus pentoxide.

The substance was very similar to the cytopentans and hemicelluloses described elsewhere. It was a white powder, soluble in water, and gave strong reduction with Fehling's solution after hydrolysis with hydrochloric acid. The presence of pentose or other furfural-yielding groups was indicated by a strong positive phloroglucinol reaction.

The entire process was repeated with a different batch of turnips and the yield of hemicellulose in each case was between 3 and 4 % of the original pectinogen.

The following analytical results were obtained:

	Ash %	Furfural %
1st product	17·16	12·04
2nd product	23·29	12·77
	Average	12·40

The high ash content of these samples is inherent in the method of preparation, as the calcium carbonate formed in the cold lime-water extract is very finely divided and difficult to separate completely.

A portion of the substance was dialysed for some days in presence of very dilute acid, reprecipitated and dried. This gave the following:

Ash: 5·1 %; Furfural yield: 12·71 %.

An important point arises from the foregoing with regard to the procedure adopted. The time of heating—24 hours—was decided on because the composition of the pectinogen appears to be more stabilised after heating for that period. We may expect the maximum amount of chemical action after such a period, and, therefore, an increased yield of any substances associated with pectinogen. There is only one element of uncertainty and that is in respect of the solubility of the hemicellulose. The extracting solution is sufficiently alkaline to liberate the methyl alcohol in the course of the extraction; is it sufficiently alkaline to liberate the hemicellulose, and is there likely to be any of this substance lost in the alcoholic filtrate after precipitation of the pectinogen?

This point was settled by the following experiments: 120 g. of cell-wall substance from turnips were heated for 24 hours with 2400 cc. of 0·5 % ammonium oxalate solution at 85–95°. The extract was filtered through a Buchner funnel and the pectinogen precipitated with 2–3 volumes of 95 % alcohol. The product was redissolved and reprecipitated, and finally dried in graded strengths of alcohol. The combined alcoholic filtrates were evaporated to small bulk under diminished pressure and lime-water added to precipitate the ammonium oxalate and any pectinogen still in solution. Air was passed through the solution while hot, to free it from ammonia, and further evaporation took place after careful filtering through fine filters to remove calcium oxalate. The concentrated liquid gave no reduction with Fehling's solution before or after hydrolysis with dilute hydrochloric acid, and the addition of alcohol and ether failed to produce any precipitate. From this we concluded that the hemicellulose was not split off under the action of the extractive, but that a stronger alkaline action was necessary.

In view of the success attained by following out the indications of the small-scale experiments (p. 688) on turnip pectinogen, it was decided to adopt the same large-scale procedure in the case of pectinogen derived from onions. The objects were to determine whether a hemicellulose was usually associated with pectinogen, and if so, whether the characteristics of such a substance were the same from different sources.

Using onions as the source, the yield was somewhat larger than that obtained from turnips. The product was prepared in an exactly similar manner to that already described. The following are average results from two samples of different origin:

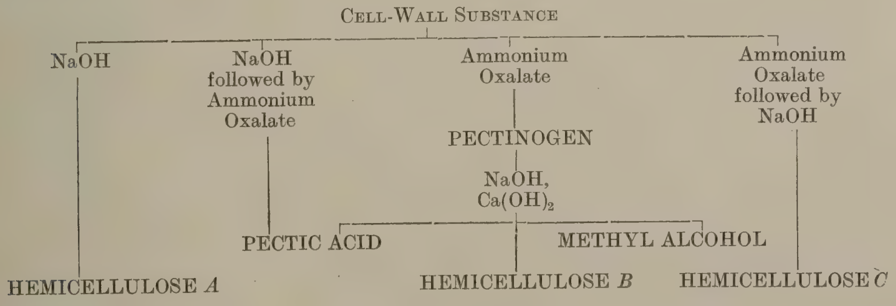
	Ash %	Furfural %
1st product	12·03	9·64
2nd product	10·76	8·62
	Average	9·13

It will thus be observed that although substances of the same general type were obtained in each case, they did not appear to be identical, as shown by furfural yields. It will be remembered that an earlier paper [Clayson, Norris and Schryver, 1921] described the cytopentans, substances similar to the hemicelluloses, and found that no agreement in analyses was to be observed in these substances prepared from a variety of sources. It is probable that they are mixtures, or substances varying with the metabolic processes in the plant. Our present knowledge does not enable us to form any rigid theories as to their nature.

Reverting to our suggested formula for the chief constituent of pectinogen, it will be seen that the slight departure from theoretical analytical results can be explained by the presence of this small quantity of furfural-yielding substance. There is therefore every reason to believe that pectinogen consists of a methylated pectic acid, in which the methoxyl groups are readily eliminated together with a small quantity of another furfural-yielding substance, similar in general character to the hemicelluloses. The conversion of pectinogen to pectic acid is not one solely of de-esterification, but involves in addition the separation of this secondary substance.

Additional note on the hemicelluloses.

Clayson, Norris and Schryver have previously shown [1921] that by direct treatment of the cell-wall substance with 4 % sodium hydroxide products similar to the hemicelluloses of Schulze were obtained. In this paper we have described the preparation of substances of the same general type by the action of alkalies on pectinogen, with which they appear to be associated. In addition, we have found that, after extracting pectinogen by the usual methods, sodium hydroxide is still capable of liberating hemicelluloses from the cell-wall substance. It will be convenient to represent these results by the following scheme:



We thus have three types of hemicellulose, and the object of further researches will be to determine the relation, if any, existing between them and the other constituents of the cell-wall.

Hemicelluloses of the type *C* were prepared by treating the cell-wall substance, from which all pectinogen had been extracted and washed out, with 4 % sodium hydroxide. The alkaline extract was neutralised with dilute acetic acid and the product precipitated with 95 % alcohol. Solution and reprecipitation followed, after which drying was carried out in the usual manner.

The table shows the furfural yields of hemicelluloses of the three types. The products were obtained as far as possible from the same batch of turnips or onions; results in which an asterisk is shown are either abstracted from a paper by Clayson, Norris and Schryver [1921] or are from different batches of raw material.

Source	*A*		*B*		*C*	
	Ash %	Furfural %	Ash %	Furfural %	Ash %	Furfural %
Turnips	5·9*	41·11*	17·16	12·04	8·18	18·39
	—	—	23·29	12·77	9·4	19·31
Onions	3·8	10·89	12·03	9·64	6·1	9·80
	2·5*	20·32*	10·76	8·62	4·3	9·28

The relationship between the various types as shown by these analyses is very slight, and it would be inadvisable to attempt any generalisation. It is hoped to conduct further enquiries into the nature of these substances at a later date.

The hemicelluloses are not simple products of the condensation of pentose and hexose groups, but contain in addition sugar acids of the galacturonic acid type as indicated by the evolution of carbon dioxide on hydrolysis in accordance with the method advocated by Nanji, Paton and Ling [1925].

SUMMARY.

1. Recent investigations on pectic substances are summarised, and indicate that pectic acid is a definite chemical substance and is, in fact, the basal molecule of this class of substance. The conception of Nanji, Paton and Ling, whereby the molecule may be regarded as a ring with six sides, these corresponding to one molecule of galactose, one molecule of arabinose, and four of galacturonic acid, the carboxyl groups of the last being free, may be accepted as a convenient working hypothesis.

2. Pectinogen occurs in the cell-wall as a methylated pectic acid in loose combination with metallic ions such as calcium. It is probable that three of the free carboxyl groups in the parent acid are methylated, leaving the remaining one to fulfil its acidic function. There are indications that an intermediate compound, containing only one methoxyl group, may be obtained, and that it is more stable than the trimethylated compound.

3. Pectinogen is obtained in the laboratory by extracting the cell-wall substance with warm 0·5 % solutions of such substances as ammonium oxalate or oxalic acid. Its composition, especially with regard to methoxyl content, varies considerably with certain factors, of which the period of extraction is the most important. The shorter the period of extraction, the more nearly does the composition of the resulting pectinogen correspond to

that of pectinogen as it occurs in the cell-wall, although the yield is not theoretical.

4. Alkalies, such as lime-water, effect the conversion of pectinogen to pectic acid, which is precipitated. The action is not one solely of de-esterification, since, in addition to methyl alcohol, a substance similar to the hemicelluloses is found in the alkaline filtrate. This hemicellulose may be an unstable or variable constituent, since its composition as shown by furfural yield, does not appear to be constant.

5. Substances of the general type described as hemicelluloses may also be obtained by direct extraction of the cell-wall substance with caustic soda or by similar treatment after extraction of pectinogen. It is difficult at present to draw any conclusions with regard to the relation of these substances to the pectic complex, as they appear to be mixtures, variable in composition, such variations being very probably due to the processes of metabolism in the plant.

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CIII. A NOTE ON THE OCCURRENCE OF A PECTIC SUBSTANCE IN BEECH WOOD.

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The microscopical examination of a disc cut from the log of a freshly felled beech tree was carried out by Mr J. F. Martley in the Forest Products Research Laboratory at South Farnborough in the spring of 1924. It showed that a tertiary layer was present in the cell-wall over certain areas of the disc. This tertiary layer, which was confined to a sector extending from 90° to 290° approximately (*i.e.* the southern side of the trunk), was distinguished from the normal secondary thickening layer of the cell-wall both optically and in its reactions to stains and reagents. It was also found to respond to the microchemical tests for pectin.

There being no evidence in the literature of the isolation and chemical examination of a pectic substance from wood, it was decided to apply to the sawdust obtained from the same beech log as the disc mentioned above, the method for the isolation of pectic acid from unlignified tissue advocated by Clayson, Norris and Schryver [1921].

For this purpose, 475 g. of sawdust from the north side of the trunk and 300 g. from the south side were treated separately. They were extracted several times with hot water, and then with 4 % sodium hydroxide solution to extract the hemicellulose [O'Dwyer, 1923]. The sawdust, after the soda solution had been filtered off, was washed free from alkalies, and then treated with hot 0·5 % ammonium oxalate solution several times, and filtered.

Although a large percentage of the tannin had been extracted from the sawdust by the treatment with hot water and sodium hydroxide, a considerable amount came out after treatment with hot ammonium oxalate, and the addition of hydrochloric acid to the ammonium oxalate extract brought down tannin as well as a white precipitate. Hence it was found necessary to eliminate tannin before adding hydrochloric acid to the solution. The reagent used for its removal was hide-powder, employed according to the methods for tannin analysis as outlined in the *Official Methods of the Society of Leather Trade Chemists*, without the preliminary light chroming. This method proved quite successful. The hide-powder did not precipitate the pectic substance, which came down afterwards on the acidification of the tannin-free solution with hydrochloric acid.

This substance, which is insoluble in water but soluble in ammonia, was purified by dissolving in 0·5N ammonia and reprecipitating with hydrochloric acid, when a thick gelatinous mass was formed. This was dialysed for some days with distilled water, until the liquid outside the dialyser gave only the faintest turbidity with silver nitrate. The substance was then treated with graded strengths of alcohol and then with ether; to complete the drying it was allowed to stand over phosphorus pentoxide in a vacuum desiccator.

It was found that the yield from the 475 g. from the north side was 1·476 g. (about 0·3 %), whilst the yield from the 300 g. from the south side of the beech log was 1·44 g. (a little less than 0·5 %). The product was found to be free from nitrogen by the method of Kjeldahl. The furfural yield was determined by hydrolysing about 0·2 g. with 12 % hydrochloric acid and treating the distillate with phloroglucinol, as described by the author in a previous communication [1923]. The test for methylpentose gave a negative result. The amount of furfural phloroglucide, reckoned as pentose, obtained from the product from the north side, was 43·0 %, and from that from the south side of the log 42·6 %. These figures approximate very closely to those obtained by Clayson, Norris and Schryver [1921] for the pentose content of pectic acid.

The optical rotation was obtained from a solution of the product in 0·5N ammonia, but, as in the case of pectic acid obtained from unlignified tissue, it was only found possible to employ small concentrations. A 0·12 % solution gave a specific rotation of + 264°, which is within the limits found for the specific rotation of pectic acid obtained from various sources.

The following results were obtained on analysis by the method of Dennstedt:

(1) 0·1936 g. gave 0·2808 g. CO_2 and 0·1008 g. H_2O.
Ash = 0·0053 g.; C = 40·73 %; H = 5·89 %; O = 53·38 %.
(2) 0·1931 g. gave 0·2820 g. CO_2 and 0·1044 g. H_2O.
Ash = 0·0065 g.; C = 40·84 %; H = 5·79 %; O = 53·37 %.

The figure obtained for carbon in each case is a little lower, and that for hydrogen a little higher than those obtained for pectic acid in unlignified tissue [Clayson, Norris and Schryver, 1921], but the differences are very slight. Owing to the small amount of the product obtained, it was impossible to purify it by the methods of these authors.

Had this been done, there seems to be little doubt that the results would have agreed even more closely with theirs.

SUMMARY.

There seems to be no reasonable doubt that the product obtained from beech wood by extraction with hot dilute ammonium oxalate and precipitation with hydrochloric acid is of a pectic nature and corresponds with the pectic acid obtained by Clayson, Norris and Schryver from unlignified tissue.

The fact that a considerable amount of the product was obtained from a part of the log in which no evidence of a tertiary layer could be found microscopically seems to show that the pectic substances are not confined to this particular layer in the cell-wall.

The isolation of this substance from lignified tissue is of some importance, as it seems probable, from other work being carried out in this laboratory, that there may be some connection between the amounts of pectic substance and of hemicellulose in lignified and unlignified tissue respectively.

The above work was made possible by a grant to the author from the Department of Scientific and Industrial Research. To the Department, to Prof. S. B. Schryver, under whose direction the research was carried out, and to Mr J. F. Martley, who undertook the microscopical examination of the disc of beech, the author's best thanks are due.

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CIV. THE ESTIMATION OF PHOSPHORUS COMPOUNDS IN BLOOD.

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A critical perusal of the literature on this subject will show that in few cases have the proposers of methods adduced any proof of their accuracy. As we were in need of a convenient method of *known* probable error we subjected the question to a rather prolonged investigation, and were able finally to establish a procedure by means of which total, inorganic, lipin[1] and (if desired) "acid-soluble" phosphorus may be conveniently estimated in blood, plasma or corpuscles with a probable error not exceeding ± 5 %.

Only two of the published methods appear to be sufficiently simple and convenient for routine use, namely, the colorimetric method described in its first form by Bell and Doisy [1920], and later modified by Briggs [1922, 1924], and the nephelometric method of Bloor [1918].

Numerous preliminary experiments with Bloor's method convinced us that the inconvenience and uncertainty of the process made it most undesirable if large numbers of analyses were to be done quickly.

Bell and Doisy's method [1920] as modified by Briggs [1922] has been used fairly extensively, but no satisfactory evidence as to its reliability has ever been furnished. Briggs [1922] talks of a "non-fading colour for comparison, the proportionality of which is exact over a wide range." In his second paper [1924] he merely states that certain suggested changes leave "the proportionality to inorganic phosphorus unimpaired." Martland and Robison [1924] remark that "there are many pitfalls in the determination of phosphorus compounds in blood," but they do not seem to suspect any lack of proportionality between the colour developed and the amount of phosphate present.

We set out to see how far Briggs' method satisfied the following criteria:

(1) Correct estimation of known amounts of phosphates in pure aqueous solutions.

(2) Agreements of duplicate analyses of the same blood or blood products.

(3) Correct recovery of known amounts of phosphate added to blood or blood products.

The method depends on the fact that the phosphomolybdic acid produced when molybdic acid is added to a solution containing phosphate is reduced with formation of a blue colour by many reducing agents, the one used by Bell and Doisy and by Briggs being quinol (hydroquinone). Since the coloration with the small amounts of inorganic phosphorus normally present in blood may be too pale for good colorimetric comparison, we tried various other reducing agents, such as amidol and metol. With metol more intense colorations can be obtained, but unfortunately they are so much less stable that we returned to quinol as reducer.

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[1] "Lipin" is used throughout in place of the old term "lipoid."

Degree of acidity. The degree of acidity of the complete mixture at the time colour begins to develop has an important influence on the depth of coloration produced by any given amount of phosphate.

With gradually increasing concentration of acid (sulphuric acid) in the final solution in which colour develops, the depth of coloration produced increases to a maximum and then decreases. Further, in the neighbourhood of the maximum the coloration is less stable than when it is paler than the maximum. The instability is manifested by a gradually increasing depth of colour terminating sooner or later in the appearance of inky-blue streaks spreading through the liquid. This change, which in the less stable solutions may become apparent in little more than an hour, obviously indicates a general reduction of the molybdic acid, but the cause of it is obscure. It always begins at the surface of the liquid, but it is not due to contact with atmospheric oxygen or to the absence of sulphur dioxide from the solution.

From these observations it is clear that it is necessary to compromise between the degree of acidity giving the maximum coloration and the degree of acidity which gives the maximum stability, because at maximum stability the coloration is too pale for satisfactory colorimetric comparison. From the behaviour of our series of mixtures we consider that it is best so to arrange matters that the final solution in which the colour is developing is $N/2$ in respect of the sulphuric acid in it.

The question of acidity must also be considered from another aspect. The phosphorus of a blood product must be brought into the form of inorganic phosphate for its actual estimation. In the case of the total phosphorus and the lipin phosphorus this is effected by digestion with sulphuric and nitric acids, and the acidity of the final solution is due solely to the sulphuric acid contained in it. But in the case of the inorganic phosphorus, the blood, plasma, or corpuscles are first precipitated with trichloroacetic acid to remove proteins, and the filtrates contain considerable quantities of that acid. Martland and Robison mention that trichloroacetic acid contains an impurity (removable by distillation) which itself slowly develops a coloration with molybdate and quinol. This we can confirm, but we cannot confirm their statement that trichloroacetic acid so purified does not affect the colour development in a mixture containing phosphate, molybdate, quinol, etc. On the contrary, it is necessary not only to balance the sulphuric acid content of standard and unknown, when the solution is a digestion product, but also to balance the trichloroacetic acid contents when the unknown is a filtrate. The balancing is best done by titrating an aliquot part of the diluted digestion product or filtrate (with sodium hydroxide and phenolphthalein) and adding the amount of sulphuric acid or of trichloroacetic acid so determined to the standard; but using the quantities detailed below in *Experimental Data* we found that the quantity of sulphuric acid to be added to the standard for a digestion product was always such as to make the final solution in which the colour develops very nearly $N/2$ in respect of this acid, and the amount of trichloroacetic acid to be added to a filtrate standard such as to make it $N/10$ in the case of a whole blood filtrate. In the case of the plasma rather more and in the case of the corpuscles rather less free trichloroacetic acid remains in the filtrate. Hence it is necessary to use the three different standards mentioned in *Experimental Data* for filtrates from whole blood, plasma and corpuscles respectively.

It will be seen that, when a product for comparison is obtained by digestion, it and its standard are $N/2$ as regards sulphuric acid, whilst in the case of a filtrate the acidity is $N/2$ in regard to sulphuric acid plus about (in all three cases) $N/10$ as regards trichloroacetic acid. As we have purposely chosen to work in a fairly stable range of the scale of acidity this difference between $N/2$ in the one case and $N/2$ plus about $N/10$ in the other does not matter, provided the appropriate standards are used. This means that in the case of a digestion product the requisite quantity of sulphuric acid must be added to the standard, and in the case of a filtrate the requisite quantities of sulphuric acid *and* of trichloroacetic acid must be added to the standard. In the latter case the same quantity of sulphuric acid must, of course, be added to the filtrate also. The most convenient way of doing this is to employ for the filtrate estimations a 5 % molybdate solution to which the requisite proportion of sulphuric acid has been added, and to use for the digestion products a neutral molybdate solution, and add to the "digestion" standard the sulphuric acid beforehand.

Proportionality of colour produced to phosphate present. It has already been mentioned that no experimental data seem to have been published on this point.

We consider that the estimations about to be referred to prove that unknown and standard should not differ by more than about 30 % if the results are to be free from uncertainty.

Preliminary experiments (four in each case), using Briggs' standard and our own, which is twice as strong, gave a maximum error of 3 % with concentration differences up to 100 %, but later experiments on blood products indicated that this agreement was fortuitous. Thirteen pairs of estimations were then made, the first of each pair employing our usual standard (except in four cases, where Briggs' standard was used) and the second of each pair using a standard nearly of the concentration of the unknown. In the first of each pair the unknown was about two and a half times the strength of the standard, and the errors ranged from + 14 % to + 7 %. In the second of each pair, where unknown and standard did not differ by more than about 10 %, the errors ranged from 0 % to − 3 %. Similar results were obtained in the recovery of inorganic phosphate added to blood filtrates.

Finally, we compared with our standard a series ranging in concentration from two-thirds of the standard by six steps up to one and a half times the standard. This series was repeated five times. The errors of all thirty estimations lay between − 2 % and + 5 %.

Our experiments on recovery of phosphate from blood products (compare next paragraph) show that the permissible difference of concentration is then frequently smaller than in the case of the pure phosphate solution, and approximates to the 25 % difference mentioned by Myers and Shevsky [1921] and confirmed by Randles and Knudson [1922] as being the limit for the original Bell and Doisy method.

Recovery of phosphate added to whole blood. Inorganic phosphate added to whole blood is quantitatively recovered (maximum observed error 5 %) if the blood plus phosphate is digested, and the added phosphate reckoned by deduction of the *total* phosphorus of the blood. If, however, the blood after addition of phosphate is precipitated with trichloroacetic acid and the added phosphate is estimated by deduction of the inorganic phosphate of the plain blood, the results are up to 13 % too high, although duplicate analyses *using the same blood* agreed within 2 % in every instance. This is the only case in which quantitative recovery (within 5 %) of added phosphate cannot be obtained. The effect is possibly due to an alteration in the p_H of the plasma caused by the added phosphate [compare Martland, 1925].

Recovery of phosphate added to plasma. In eight experiments the errors ranged from + 1 % to + 4 %.

Agreement of duplicate analyses of inorganic phosphorus in whole blood. The bloods were laked with 1 % trichloroacetic acid as described by Martland and Robison. Fifteen samples were dealt with, from each of which two separate filtrates were made. The differences between duplicates ranged from 0 % to + 6 % (in one case).

General conclusions as to the estimation of inorganic phosphate.

We think it follows from the experiments above described that small quantities of inorganic phosphate may be recovered from pure phosphate solutions, from plasma or plasma filtrate, and from blood filtrate with a probable error of not more than 5 % provided that:

(1) Approximately equal concentrations of the colour-producing reagents and approximately equal temperatures are maintained.

(2) The specified degree of acidity exists in the solutions at the time the colour is developing and that it is the same in both solutions.

(3) The phosphate concentrations of unknown and standard do not differ by more than about 30 %.

(4) Where trichloroacetic acid is present in the unknown it is balanced by an equal amount in the standard.

Inorganic phosphate added to *whole blood* before precipitation is not quantitatively recovered within this limit, probably owing to changes caused by the added phosphate itself, but the agreement of duplicate analyses of any particular blood, with or without added phosphate, is as good as that observed with pure phosphate solutions, plasma, or with blood or plasma filtrates.

Improved digestion process for total and lipin phosphorus.

For the estimation of total phosphorus, lipin phosphorus and "acid soluble" phosphorus (*i.e. total* phosphorus of a filtrate) the phosphorus must be brought into the form of inorganic phosphate. This has been usually effected by means of digestion with a mixture of concentrated sulphuric and nitric acids (H_2SO_4 one part, HNO_3 two parts), but many references are to be found in the literature to difficulties which have been met with in carrying out such digestions. These troubles arise chiefly from the fact that whilst it is necessary to destroy all the organic matter and to obtain a final product absolutely free from nitric acid—these considerations require a fairly high temperature—yet if the temperature is too high not only is sulphuric acid lost (which upsets the final degree of acidity) but phosphoric acid may volatilise with it, as Baumann [1924] has shown. For this reason Baumann proposes to replace the nitric acid by concentrated hydrogen peroxide. We have not tried this, as with the procedure about to be described the nitric acid digestions need give no trouble.

The heat applied should be so regulated right from the start that it is just sufficient, when all the water, etc. has boiled off, to keep the remaining small quantity of sulphuric acid just at or below its boiling-point. Using the ordinary micro-burners which have been generally recommended it is very difficult to hit upon or to maintain precisely this measure of heat. In our experience to run two digestions simultaneously requires the continuous attention of a skilled person for a considerable time, and even then losses from overheating occasionally happen.

Electrical heater. This uncertainty and error and the tedious personal attention can be entirely eliminated by the use of a suitable electrical heater. With such a heater it is also possible to carry out any number of digestions simultaneously.

The instrument was improvised from the heating elements of an ordinary electric radiator which is on the market[1], and which by a fortunate chance might have been designed for the purpose.

The heating elements referred to consist of porcelain troughs $5\frac{1}{2}$ inches long and $1\frac{1}{8}$ inches wide divided by lateral porcelain partitions (the whole is in one piece) into six small square compartments each measuring about $\frac{3}{4}$ inch square (internal) at the top. The partitions are pierced with holes through which the spiral resistance wire passes. The wire is in this way suspended about the middle of the trough. For the purpose of these digestions it is necessary that the bottoms of the pyrex tubes should be just in contact with the hot spiral, but it will be found that in the case of the usual 250 × 25 mm. tubes the squares are slightly too small to permit of this. The spiral is therefore removed and the internal edges of the square cautiously chamfered off (by holding them against a carborundum stick rotated in the lathe) until, on trial, the tube is found just to touch an inserted twist drill or similar object of the same diameter as the spiral. The heating element thus modified is mounted on a sheet of uralite which is separated by two half-inch pieces of wood from a light wooden base-board. The heater is then placed between the end pieces of a light wooden test-tube rack about 10 inches high, the top of which is bored with $1\frac{1}{8}$ inch holes in positions corresponding with the square compartments of the porcelain below. The instrument is connected in series with a sliding rheostat of suitable resistance.

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[1] The radiator in question is known as the Revo, and is made by the Cable Accessories Company, Tividale, Staffs. A set of four heating elements each containing six compartments can be purchased for 12*s*.

When all the more volatile matter has boiled off from a digestion there is left about 0·3 cc. or 0·4 cc. of sulphuric acid. It is necessary to find by trial that setting of the rheostat which will give the heat required to keep this small quantity of acid just in the neighbourhood of its boiling-point, so that the production of only an occasional bubble of gas is to be noticed in the liquid. Once this point has been found, and given a reasonably constant voltage, the apparatus needs no further attention and is merely switched on every time a new batch of tubes is introduced.

If the degree of heat applied is that specified above there will usually still be nitric acid present when the transitory production of dense fumes indicates the departure of the last traces of water, for the fumes will be brown. If the liquid is colourless, it is only necessary to continue the heating until no brown fumes are to be seen, and then add the sugar solution mentioned below. The liquid may, however, still be brown. In this case it is of no use to continue heating after the brown fumes have disappeared. The current should be cut off, and when the tubes are nearly cold one or more drops of concentrated nitric acid should be added and the heating continued.

Addition of sugar. If the process is conducted as described no loss of sulphuric acid or phosphoric acid by volatilisation can occur, because after the water has disappeared all the vapours condense within the tube; but there is another possibility that must be guarded against. Bloor [1918] obtained low results if the digestion was stopped at this stage, and ascribed them to the presence of some combination of phosphoric and nitric acids which did not react with strychnine molybdate. Martland and Robison [1924] sometimes obtained low results, which they considered due to absorption of phosphoric acid by the glass in consequence of overheating. In any case, nitric acid or its decomposition products frequently remain in the product even after prolonged heating subsequent to the disappearance of brown fumes, for they can be smelt in the cold digestion product. Whether they bind any phosphoric acid or not, these nitrogen compounds must be removed, because they upset the colorimetry. When the digestion product is colourless and no more brown fumes are visible we therefore (following Bloor) add as many drops of 1 % cane sugar as will cause a distinct browning of the partially cooled but still hot liquid. The current is then switched on and the heating continued until the liquid is colourless again.

EXPERIMENTAL DATA.

We give below the quantities to be taken for the various blood phosphorus estimations in accordance with the results which have been described above. The actual quantities mentioned are based on the obtaining of 15 cc. of the final blue solution of a depth of colour suitable for comparison in a 25 mm. cell in the dilution colorimeter [Stanford, 1923]. Should any other final volume be desired it is important to note that the concentration of *all* the substances present in that final solution in which the colour is developing must be that here given.

1. *Reagents*[1].

(*a*) Acid molybdate (for inorganic phosphorus in blood, plasma or corpuscles).

Dissolve 25 g. of ammonium molybdate in 300 cc. of water, then add 75 cc. of concentrated sulphuric acid previously diluted with 125 cc. of water.

(*b*) Neutral molybdate (for total, lipin and "acid soluble" phosphorus) is a filtered 5 % solution of ammonium molybdate.

(*c*) Quinol 1 % solution.

(*d*) Sodium sulphite 20 % solution.

2. *Standards for inorganic phosphate.*

	(*a*) For whole blood	(*b*) For plasma	(*c*) For corpuscles
KH_2PO_4 (1 cc. ≡ 6·0 cmgm. P)	72·0 cc.	72·0 cc.	72·0 cc.
$CCl_3.COOH$ (20 % solution)	74·4	91·2	57·6
Water	357·6	340·8	374·4
	504·0	504·0	504·0

3. *Estimation of inorganic phosphates.*

Mix 20 volumes of blood, plasma, or corpuscles with 60 volumes of 1 % trichloroacetic acid, then add 17 volumes of 20 % trichloroacetic acid and 3 volumes of water. Shake vigorously, and allow to stand for a few minutes before filtering. To 10 cc. of the filtrate add 0·5 cc. of a KH_2PO_4 solution containing 8 cmgm. of P per cc. (This 4·0 cmgm. is of course to be deducted when calculating the result.) To the 10·5 cc. of solution so obtained 1·5 cc. each of *acid* molybdate, quinol and sulphite are successively added, making a total volume of 15 cc. To 10·5 cc. of the appropriate standard (see above,

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[1] The abbreviation "cmgm." stands for "hundredth of a milligram."

2 (*a*), (*b*) or (*c*)) 1·5 cc. each of *acid* molybdate, quinol and sulphite are added. The additions of molybdate, etc. to the two solutions should be as nearly simultaneous as possible, and the colorimetric comparison should be made after 30 minutes.

4. *Standard for total, lipin and "acid soluble" phosphorus.*

KH_2PO_4 (1 cc. ≡ 6·0 cmgm. P)	72·0 cc.
H_2SO_4 (10*N*)	36·0
Water	396·0
	504·0

5. *Estimation of total phosphorus.*

Take of whole blood 0·4 cc., or of plasma 1·5 cc., or of corpuscles 0·25 cc. (previously laked with an equal volume of warm water), and digest with 1 cc. of a mixture of concentrated sulphuric acid (1 part) and concentrated nitric acid (2 parts), adding three small pieces of quartz to prevent bumping. After digestion make up to 15 cc. in a graduated tube. Of each digestion product take 10 cc., add 0·5 cc. of water, followed by 1·5 cc. each of *neutral* molybdate, quinol and sulphite. The standard solution simultaneously set up contains 10·5 cc. of the standard for total, etc. phosphorus given above, paragraph 4, and 1·5 cc. each of *neutral* molybdate, quinol and sulphite.

6. *Estimation of lipin phosphorus.*

Take of whole blood 1·5 cc., or of plasma 2·0 cc., or of corpuscles 1·0 cc. (previously laked with an equal volume of warm water). In each case add drop by drop to about 20 cc. of a mixture of alcohol (redistilled) 3 parts and ether (redistilled) 1 part. Heat to boiling in a water-bath, cool, filter through a small paper into a pyrex tube (250 × 25 mm.) and boil off the solvent in the water-bath. The blood product is repeatedly washed with small quantities of the extraction mixture, the washings being filtered into the pyrex tube and boiled off. The tube is finally left in boiling water for a few minutes. The acid digestion mixture (1 cc.) is then added and the subsequent procedure is the same as that for total phosphorus.

SUMMARY.

The conditions are described which are necessary for obtaining accurate results in the estimation of inorganic phosphate by the colorimetric method.

The process of digestion with acids used for bringing total P or lipin P into the form of inorganic phosphate has also been studied, and an electrical heating device is described.

Methods are given in detail for the estimation of inorganic, total, lipin and "acid soluble" P in blood, plasma and corpuscles, and evidence is presented that the results are accurate to within a probable error of 5 % of the amount of phosphorus estimated.

We are indebted to our colleagues, Dr J. Walker and Dr S. Grossman, for the collection of the very numerous samples required for this investigation.

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CV. THE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN BLOOD.

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(*Received June 15th, 1925.*)

It follows from the work of numerous observers that phosphorus is to be found in blood as inorganic phosphate, as lipin phosphorus compounds and in other forms of combination unknown: the last is frequently referred to as the "unknown" P. Bloor [1918], using his nephelometric method, seems to have been the only one to attempt a general survey of the question. Other statements in the literature are of a more or less fragmentary and disjointed character. For various reasons we do not consider that the figures given by Bloor as a result of his extensive investigation can fairly be regarded as final.

After we had elaborated, therefore, the method with known limits of error described in the preceding paper we proceeded to investigate a few human bloods for purposes of orientation. Five individual bloods were obtained, and in each case the following estimations were made on the whole blood, the plasma and the corpuscles, namely, total P, inorganic P, lipin P and, by difference, "unknown" P. We also included the "acid soluble" P, because it has so frequently been the object of estimation by previous workers. The results are given in Table I (p. 707).

All figures are cmgm. (*i.e.* hundredths of a milligram) of P per cc.

Analysis of results.

From cases 1, 2, 3, 4 and 5 it appears that the phosphorus compounds of the blood are divided on the average as shown in Table II.

The calculations have been made on the basis of the calculated value for the whole blood "total" derived from the ascertained corpuscle percentage, and not on the experimentally determined whole blood "total." From Table I it will be seen that the agreement between the calculated and observed values is in most cases pretty good.

Lipin phosphorus. The "acid-soluble" P is the total P of the trichloroacetic acid filtrate. On the assumption that the lipin P compounds are precipitated with the proteins, and that no other P compounds are, and that any P in protein combination will be negligible in amount it follows that the figures for lipin P would be equal to the difference between total P and "acid-soluble" P. The figures relating to this point, calculated from the experimental results in Table I, are given in columns 6 and 7 of that table. The 7th column shows

Table I.

Distribution of P in Human Blood

		1 Total	2 Inorganic	3 Lipin	4 "Unknown" by difference	5 "Acid-soluble"	6 Total minus "acid-soluble"	7 % difference
1	Whole blood	40·5	2·7	11·0	26·8	Not estimated	—	—
	,, (calculated from P + C)	35·9	2·4	9·7	—	25·9	—	—
	Plasma	10·7	2·9	7·3	0·5	3·0	7·7	5
	Corpuscles (49·2 %)	62·0	1·9	12·2	47·9	49·5	12·5	2
2	Whole blood	38·1	3·9	12·5	21·7	25·4	12·7	1
	,, (calculated from P + C)	36·0	3·1	10·6	—	23·6	—	—
	Plasma	12·6	3·7	8·9	0·0	3·7	8·9	0
	Corpuscles (42·4 %)	67·8	2·2	13·1	52·5	50·9	16·9	30
3	Whole blood	32·8	2·8	8·5	21·5	23·4	9·4	9
	,, (calculated from P + C)	31·6	2·2	8·3	—	—	—	—
	Plasma	6·7	2·3	4·1	0·3	2·0	4·7	15
	Corpuscles (47·1 %)	59·4	2·0	12·9	44·5	Not estimated	—	—
4	Whole blood	39·5	3·8	11·5	24·2	25·0	14·5	25
	,, (calculated from P + C)	36·6	3·2	10·9	—	24·6	—	—
	Plasma	12·2	3·8	8·1	0·3	3·8	8·4	4
	Corpuscles (43·8 %)	68·0	2·4	14·5	51·1	51·3	16·7	15
5	Whole blood	33·0	2·0	10·0	21·0	21·6	11·4	14
	,, (calculated from P + C)	33·9	2·0	9·1	—	—	—	—
	Plasma	7·9	1·8	5·6	0·5	Not estimated	—	—
	Corpuscles (49·0 %)	61·0	2·3	12·7	46·0	40·9	20·1	60

Table II.

	% of total P	Mean of
In the plasma		
Inorganic	5	4, 6, 4, 6, 3
Lipin	10	10, 14, 7, 12, 9
"Unknown"	0	0·6, 0·0, 0·5, 0·8, 0·6
Total	15	15, 20, 11, 19, 13
In the corpuscles		
Inorganic	3	2, 3, 3, 3, 3
Lipin	17	17, 15, 19, 17, 18
"Unknown"	65	66, 62, 67, 61, 67
Total	85	85, 80, 89, 81, 88

the percentage difference between column 5 and column 3, the percentage being reckoned on the latter.

It will be seen that the total "acid-soluble" is never less than the experimentally determined lipin. The differences range from 0 % to + 30 % (in one case 60 %). Two explanations are possible for this. Whilst it was shown in the preceding paper that the methods employed have a probable error not exceeding 5 %, yet there is the limitation in the case of the lipin P that the extraction process with alcohol and ether, although customary, is empirical. It is assumed that it extracts the lipin P compounds completely, and nothing else. Apart from this, the extraction of a mass of protein precipitate with an alcohol-ether mixture excites suspicion as a quantitative procedure. It is therefore doubtful whether the differences found are due to incomplete extraction, or to the presence of P compounds which are precipitated by trichloroacetic acid, but are not soluble in an alcohol-ether mixture.

General Conclusions.

From the analysis of results in Table II it appears that the relative quantities of inorganic, lipin and "unknown" P in the blood are fairly constant. It is also interesting that the "unknown" P is almost if not entirely confined to the corpuscles. This has been frequently suspected before, but so far as we are aware, no very satisfactory evidence for the statement has been given.

The Phosphorus of Cerebrospinal Fluid.

In the six experiments shown in Table III, the values obtained for inorganic and total P of cerebrospinal fluid (withdrawn from the living subject) are practically identical. It is curious that the "total" figures are in each case just greater than the inorganic, but the differences are really almost within the limits of experimental error (5 %) which we have shown to be attached to the methods of estimation. At least 95 % of the phosphorus of the cerebrospinal fluid is therefore present in the form of inorganic phosphate.

Table III.

Case No.	Inorganic	Total
1	1·47	1·54
2	1·27	1·33
3	1·52	1·65
4	1·51	1·61
5	1·68	1·81
6	1·57	1·62

A part of the expenses of the work has been borne by the Medical Research Council, to whom our thanks are due.

REFERENCE.

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CVI. THE ESTIMATION OF CALCIUM IN BLOOD.

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For the estimation of the small amounts (of the order of 10 cmgm.[1] per cc.) of calcium in blood numerous methods and modifications have been described. Of these, the most convenient are those in which the calcium is precipitated as oxalate, and estimated, after solution in sulphuric acid, with permanganate. We propose to detail a short procedure which enables calcium estimations on blood or blood products to be carried out in this way with a probable error not exceeding 5 %.

Preparation of material. Precipitation with trichloroacetic acid removes the proteins without removing appreciable quantities of calcium. When the proteins are not precipitated, the calcium oxalate precipitate contains considerable quantities of organic matter, which use up permanganate.

Precipitation of calcium oxalate. The conditions in which the calcium should be precipitated have been repeatedly studied, and it has been shown that certain limits of p_H must be observed. Precipitation with oxalic acid in the presence of a definite amount of hydrochloric acid and sodium acetate has the advantage that at the concentrations concerned magnesium is not likely to be precipitated as well. The necessary conditions for the oxalic acid precipitation, originally suggested for use on blood by McCrudden [1912], are stated in a paper by Widdows [1923].

Calcium oxalate is appreciably soluble in water. This point has been noticed by Cameron and Moorhouse [1925] in the case of their precipitation in ammoniacal solution, for they calculate the amount lost in washing, but it has escaped the attention of Widdows and others who have used the direct precipitation with oxalic acid and have washed the precipitate with water.

Using the procedure of Widdows (which is in other respects entirely satisfactory) the solution at the time the calcium is precipitated contains a large excess of oxalic acid and has a p_H calculated to prevent any calcium oxalate from remaining in solution. After centrifugalisation a certain (but unstated) amount of this solution remains, and this is then diluted by the water of the first washing; what the solubility of oxalate in this mixture may be it is impossible to calculate. The same applies to the second washing, but as we are then dealing with nearly pure water the solubility may very likely be that of calcium oxalate in water as determined by Richards, McCaffrey and Bisbee [1901].

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[1] The abbreviation "cmgm." stands for centimilligram, the hundredth part of a milligram.

Another point which Widdows and others, who have washed the calcium oxalate precipitate by centrifugalisation, have not referred to is the proportionate amount of the liquid remaining in the centrifuge tube after each decantation. Relative to the minute oxalate precipitate the supernatant liquid in the first stage contains an enormous quantity of oxalic acid, and measurement of the undecanted residue is necessary before it can be decided how many washings are enough. The amount of residual liquid will depend on the shape of the centrifuge tube and on the method of decantation. Our own preliminary experiments using Widdows' method gave apparently correct results (4 % to 7 % too high) after two washings, but results 18 % to 21 % too low after three. This was because in those particular centrifuge tubes the amount of residual liquid was comparatively large, and so the free oxalic acid remaining after the second washing was enough to balance the loss due to solution of the oxalate precipitate, and so mask the effect, which, however, became apparent after the next washing. If those centrifuge tubes had been so designed as to permit of the removal of all but a fraction of a cc. of the supernatant liquid, that error would certainly have appeared at the second washing.

The remedy is to wash the precipitate of calcium oxalate not with water but with a saturated solution of calcium oxalate. This is prepared by shaking precipitated and carefully washed calcium oxalate with water and filtering through two or three thicknesses of No. 30 Whatman paper. The saturated solution so obtained must be titrated with $N/100$ permanganate to make sure that no solid particles of oxalate have passed through the filter. Richards [Richards *et al.* 1901] refers to the difficulty of filtering this solution. For this purpose it is sufficient if 15 cc. of the solution require no more than 0·20 cc. to 0·24 cc. of $N/100$ permanganate. The correct figure at 18° is 0·17 cc.

Method of estimation.

(1) $N/100$ *potassium permanganate.* The instability of very dilute solutions of permanganate is well known, and we would therefore draw attention to the method of preparation given by Halverson and Bergeim [1918]. Made in this way we have found $N/100$ permanganate not to change its titre for weeks.

(2) *Centrifuge tubes.* The amount of residual liquid after each centrifugalisation is reduced to a minimum by conducting the precipitation and washings in centrifuge tubes which are about 85 mm. long and 30 mm. in diameter, and taper evenly from about the middle to a blunt point of about 8 mm. internal diameter. The supernatant liquid is removed by means of a small syphon made of capillary tubing, and having its tip drawn out and bent upwards, so that the direction of flow is downwards. With these tubes and this syphon the amount of residual liquid is less than half a cc. so that, as may be proved by calculation, no measurable quantity of soluble oxalate can be left after the initial decantation plus two washings.

(3) *Method.* 5 cc. of plasma are precipitated with 25 cc. of trichloroacetic acid (6·5 %). Of the filtrate 20 cc. are placed in a centrifuge tube. The tube

is supported on a small wire tripod and the small stirrer previously described in connection with the dilution colorimeter [Stanford, 1923] is introduced into the liquid. With the stirrer rotating there are added firstly a drop of methyl red, then concentrated ammonia in drops until the liquid is just alkaline, then $N/2$ hydrochloric acid in drops until the liquid is just acid again. The solution so prepared is treated successively with 1 cc. $N/2$ hydrochloric acid, 1 cc. 4 % oxalic acid and 1 cc. 20 % sodium acetate. The mixture is allowed to stand overnight, and is then centrifugalised and the precipitate washed twice by centrifugalisation with the saturated calcium oxalate solution already described, about 25 cc. being used each time. The oxalate precipitate is finally dissolved in 4 cc. of N sulphuric acid, and titrated with $N/100$ permanganate.

For accurate titrations it is necessary to use a burette having a very fine tip. Ours is of 5 cc. capacity, graduated in fiftieths, and the size of drop is about 0·025 cc. As the titration figures are usually from 1·5 cc. to 2·0 cc. the titration may be regarded as being in itself accurate to about 1 % if the observer has good colour perception.

After centrifugalisation it will always be found that, whilst practically all the precipitate is compactly at the bottom, yet there are minute traces floating on the surface of the liquid. During the syphoning these traces tend to adhere to the limb of the syphon as the liquid descends. For this reason the syphon should be turned off just before the liquid meniscus reaches its intake, and the syphon limb carefully washed down with a jet of the saturated calcium oxalate solution. In trial experiments we found that after the complete set of three decantations traces of oxalate could be dissolved off the syphon limb and on titration were found to be equivalent to 0·06 cc. of permanganate. On the other hand, it is practically impossible to get reagents absolutely free from substances that attack permanganate, and we found in blank experiments on our reagents that such impurities represented, in the quantities used in an estimation, about the same quantity of permanganate (actually 0·05 cc.). We therefore allow these two errors to cancel one another by disregarding both of them.

Purity of calcium oxalate precipitate. Hirth and Klotz [1923] have stated that the calcium oxalate precipitate may be contaminated with organic matter derived from the blood, which uses up permanganate and gives too high results. To satisfy ourselves on this point, we estimated the calcium in several plasma filtrates firstly by the permanganate method above described and secondly by converting the oxalate precipitate into oxide and estimating that acidimetrically. The latter figures were certainly in all cases slightly lower (4 %) than the permanganate results, but this difference is probably due to losses taking place in the additional transferences of material from vessel to vessel necessitated by the acidimetric procedure. This is confirmed by the fact that in two experiments on the estimation of known amounts of calcium in pure $CaCl_2$ solutions, taking quantities similar to those found in blood, the acidimetric method gave results which were from 3 % to 7 % too low.

Estimation of known calcium solutions. Four consecutive estimations on standard $CaCl_2$ solutions containing about the same amount of Ca as that present in plasma gave the following errors: – 2 %, + 1 %, + 2 %, + 2 %.

Recovery of added calcium. Known amounts of calcium chloride added to plasma[1] before the precipitation with trichloroacetic acid are quantitatively recovered with a probable error of about 5 %. The errors in seven consecutive experiments were 3 %, 1 %, – 2 %, – 5 %, 2 %, 2 % and 6 %.

DISTRIBUTION OF CALCIUM COMPOUNDS IN THE BLOOD.

As after the experiments above described we were in possession of a method of a proved degree of accuracy of about 5 % we thought it well, for purposes of orientation, to examine a few human bloods from this point of view. In each case an estimation was made on the whole blood, on the plasma, and on the corpuscles. In addition the percentage of corpuscles was determined (by weighing) so that the value for the whole blood calculated from those found for the plasma and corpuscles could be compared with the value actually found for the whole blood. The results in the five cases we examined are shown in the following table, and we think that, considering the necessary limitations of micro-quantitative methods, the agreement between the observed figures for whole blood in the first line and the calculated ones in the fourth is a further indication of the reliability of the method.

It will be observed that the quantities of calcium in the corpuscles are very much smaller than those in the plasma. Kramer and Tisdall [1922] found no calcium in the corpuscles, but we consider the amounts recorded below are definitely outside our limits of experimental error. They are doubtless high from adherent plasma, but it would require a 20 % plasma contamination to account for the whole of the apparent corpuscle calcium in this way.

Table I. *Distribution of calcium compounds in blood.*

(All figures are cmgm. of Ca per cc.)

	Case 1	Case 2	Case 3	Case 4	Case 5
Blood	6·7	6·3	6·4	5·6	7·1
Plasma	11·0	9·6	10·6	10·0	10·0
Corpuscles	2·0	3·1	1·4	3·2	2·2
Blood (calculated from P+C)	6·4	6·7	6·0	6·3	6·5

The actual estimations were performed as follows:

Blood. 6 cc. blood are pipetted into a small flask, the pipette is washed out with 6 cc. of cold water, the flask is corked and placed in a warm water-bath (40°) for about 20 minutes, and shaken from time to time. After laking is complete 10 cc. are run drop by drop into 25 cc. of 6·5 % trichloroacetic acid with constant mixing. The contents of the flask are shaken well and after standing about 30 minutes are filtered through a No. 30 Whatman paper.

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[1] Anticoagulant: 3 mg. lithium citrate per cc. of blood [compare Briggs, 1923].

Plasma. 5 cc. plasma are added to 25 cc. of 6·5 % trichloroacetic acid and then treated as for blood above.

Corpuscles. 6 cc. corpuscles are laked as in the case of the blood. 10 cc. of the laked corpuscles are added to 25 cc. of 6·5 % trichloroacetic acid and then treated as for blood above.

From each filtrate 20 cc. are taken for the precipitation of the calcium.

SUMMARY.

Calcium in blood or blood products may be estimated with a probable error of ± 5 % by precipitating the proteins with trichloroacetic acid, precipitating the calcium as calcium oxalate in certain conditions as to p_H and then estimating the calcium by dissolving in acid and titrating with permanganate. The precipitate must be washed with a saturated solution of calcium oxalate, and not with water in which it is appreciably soluble.

Using this method a series of estimations on the calcium content of blood, plasma and corpuscles has been made with the results set out in Table I above.

We are indebted to our colleagues, Dr J. Walker and Dr S. Grossman, for the collection of the blood samples required for this investigation.

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CVII. ESTIMATION OF ALLANTOIN IN PRESENCE OF URIC ACID, CREATININE AND AMINO-ACIDS.

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(*Received June 5th, 1925.*)

In a study of the excretion of uric acid and allantoin in normal and diabetic dogs we found it of importance to know if higher values than usual of uric acid, creatinine and amino-acids were of any influence on the results of the allantoin estimations.

The method used for the estimation of allantoin was that of Wiechowski [1908, 1910, 1913]. The principle of the method is as follows: the urine is precipitated with phosphotungstic acid and basic lead acetate; HCl is removed and the heavy metals precipitated; in the last filtrate the allantoin is precipitated at neutral reaction by 1 % mercuric acetate solution in 20 % sodium acetate.

The estimation of allantoin is then accomplished either by estimating the nitrogen in the precipitate, or by treating the precipitate with H_2S, whereby the allantoin is set free; after crystallisation the allantoin is weighed.

The following estimations have been made:

	Allantoin estimated by the N in the Hg-precipitate (g.)	Allantoin weighed after crystallisation (g.)
(1) 0·1 g. allantoin 50 cc. of urine containing 0·02 g. uric acid	0·099	0·089
(2) 0·1 g. allantoin 0·1 g. creatinine 50 cc. human urine containing 0·02 g. uric acid	0·098	0·091
(3) 0·1 g. allantoin 0·1 g. alanine 0·1 g. leucine 50 cc. human urine containing 0·02 g. uric acid	0·161	0·091
(4) 0·1 g. allantoin 50 cc. distilled water	0·092	0·087
(5) 0·1 g. allantoin 0·1 g. alanine 0·1 g. leucine 50 cc. distilled water	0·133	0·089
(6) 0·1 g. allantoin 0·01 g. alanine 0·05 g. leucine 50 cc. distilled water	0·099	0·090

In each case the solution also contained 50 cc. of distilled water, 5 cc. 1 % H_2SO_4 and 5 cc. 50 % acetic acid.

It appears from these results that the estimation of allantoin by Hg-precipitation gives lower values in water solution than in urine (4 and 1). In urine the whole amount of allantoin apparently is recovered (1). This result, however, must be due to precipitation of other nitrogenous constituents than allantoin, as appears from (3) and (5), where apparently more allantoin is recovered than the amount which was in the solution. Even when present in small amounts other nitrogenous substances have a marked influence as appears from a comparison between (6) and (4).

The results of estimation by weighing are constant, but about 10 % too low, which may be due to loss in crystallisation.

CONCLUSIONS.

It may be concluded from these results that the estimation of allantoin by Hg-precipitation gives figures for the amount of allantoin in aqueous solution, not more than 10 % below the real amount.

In presence of amino-acids in small quantities, and of creatinine and uric acid in quantities such as are contained in normal urine, the figures for allantoin by the same method are higher and closer to the real amount of allantoin.

When amino-acids are present in larger amounts (0·2 %) the figures for allantoin are too high.

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CVIII. THE EXCRETION OF PURINE DERIVATIVES IN DOGS.

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(*Received June 5th, 1925.*)

It is generally assumed as a fact that allantoin is the terminal product of purine metabolism in dogs and that allantoin excretion in dogs has the same significance as uric acid excretion in man.

The difference between the purine metabolism in dogs and in man is believed to depend upon the presence in the dog's liver of the enzyme uricase, which oxidises uric acid to allantoin, and the lack of this enzyme in the human liver and in other human tissues [Wiechowski, 1907, 1908].

According to this conception the allantoin excretion in dogs and the uric acid excretion in man ought to have the same relative magnitude, if the only difference in their purine metabolism were that the terminal product in man was uric acid and in dogs allantoin.

It appears however from the literature concerning determination of allantoin in urines of dogs, that the excretion in many dogs is very large compared with the excretion of uric acid in man.

In papers by Wiechowski [1907, 1908], Schittenhelm [1909] and Hirokawa [1910] numerous determinations of the allantoin excretion in dogs are found. If the allantoin nitrogen in percentage of total N is calculated from their tables, it is evident that this percentage is very high compared with the uric acid N in percentage of total N in man. The percentage varies between 7 and 11.

Hunter, Givens and Guion [1914] point out this high ratio of allantoin to the total N excretion and emphasise that the allantoin output under ordinary conditions of diet or even in starvation may be so great as to account for nearly one-tenth of all the nitrogen excreted.

In man the excretion of uric acid N in percentage of total N is much smaller, the average being about 1 % if on purine-free diet.

The amount of uric acid excreted by dogs is almost always very small and the amount of other purine bases in the urine is insignificant.

In Dalmatian dogs, however, S. R. Benedict [1917] found a quite considerable excretion of uric acid, a finding which is confirmed by Wells [1918], Onslow [1923] and by Folin, Berglund and Derick [1924].

Onslow [1923] studied the inheritance of this anomaly in two generations of offspring from a Dalmatian and a terrier, and found that the anomaly was inherited according to the Mendelian law.

In his papers, however, attention was directed only to the uric acid excretion. A series of allantoin estimations was made, but no remark was passed about the very large excretion of allantoin in all the dogs, which according to the tables in many dogs amounts to more than 20 % of the total N.

When this very large excretion of allantoin in dogs is compared with the uric acid excretion in man, it is evident that the purine metabolism in the dog and in man must be quite different not only as regards the character of the terminal product.

In most of the previous investigations of the allantoin excretion in dogs, however, it is difficult or impossible to find out how much of the allantoin in the urine is due to the purine content of the food. The diet is often described only as "liberal" and "mixed." In one investigation by Harding and Young [1919] the quantity and quality of the diet is exactly described (meat and placenta), but the authors make no attempt to calculate how much of the allantoin excreted is due to the exogenous purines. It appears, however, from their tables, when we calculate the excretion of allantoin N in percentage of the total N, that on meat diet there has been an excretion of allantoin N varying between 4·3 and 9 % of the total N excretion. On placental diet the allantoin excretion has been 9–14 % of the total N.

The authors draw attention only to the difference between the output of allantoin on meat diet and on placental diet and conclude from these comparative feeding experiments that the comparatively large amount of arginine present in the placental diet is responsible for the increase in purine metabolism, thus confirming the conclusion of Ackroyd and Hopkins [1915] who in their experiments on rats found that arginine and histidine may serve as substrate for allantoin formation in this animal. Harding and Young do not make any remark on the large excretion of allantoin on meat diet, for which the purine content of the food can only be responsible to a certain limit.

Findings of large amounts of allantoin in dog's urine are very important on account of conclusions drawn from comparative studies of purine metabolism in man and dogs, and based upon the assumption that the allantoin excretion in dogs has the same significance as the uric acid excretion in man. Folin, Berglund and Derick [1924] in their studies of the destruction of intravenously injected uric acid in the dog and man, are aware of the fact that the allantoin excretion in dogs is unreasonably large, though they have no experiments bearing on this phenomenon. They remark (p. 400) that "from the standpoint of tissue metabolism, the finding of several times as much 'endogenous' purine derivatives as in man, in animals whose creatinine excretion is of the same order as that of man, points to some important missing link in the current, accepted concepts. Either the magnitude of purine metabolism in man is hidden by very extensive destruction of purine materials, or the purine derivatives, including allantoin, found in the urine of other animals have a double origin, as in birds."

In order to obtain experimental evidence for or against the probability of such a double origin of the allantoin and uric acid excretion in dogs, we have made a series of feeding experiments in which we have determined the total N, allantoin and uric acid excretion on protein-free diet, on purine-free protein diet and on high and low protein diet containing known amounts of purines.

The dogs, all mongrels, were from the time they were puppies fed on bread exclusively—no meat.

The urine was collected during 24 hours. The nitrogen was estimated by the Kjeldahl method, uric acid by the method of Folin and Wu [1919], allantoin by the method of Wiechowski [1913].

Table I.

I. *Dog A. Female, weight* 9·50 *kg.* 120 *g. fat only.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
4. x.	1·73	Trace	0·85	—	0·30
5. x.	2·56	,,	1·00	—	0·35
Average	2·14		0·92	—	0·33

15·5 % of total N

II. *Dog A.* 200 *g. horse-flesh* + 100 *g. fat.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
12. x.	5·97	79	1·84	26	0·64
13. x.	5·38	110	1·40	37	0·49
14. x.	5·02	80	1·28	26	0·45
15. x.	5·05	80	1·32	26	0·46
16. x.	5·97	107	1·94	36	0·68
Average	5·47	91	1·55	30	0·54

10 % of total-N

III. *Dog B. Male, weight* 8·90 *kg.* 500 *g. horse-flesh.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
10. vi.	15·48	164	2·46	55	0·86
11. vi.	15·88	215	2·10	72	0·74
12. vi.	16·40	302	2·37	101	0·83
Average	15·65	227	2·30	76	0·81

5·6 % of total N

It appears from I that the excretion of allantoin N in the dog on a pure fat diet is 15·5 % of the total N excretion, a percentage which is 15 times as high as in man on a fat diet.

From II and III it is seen that both the allantoin and the uric acid excretion increase when protein is given. Findings of 10 % and 5·6 % of the total N as allantoin N and uric acid N when feeding horse-flesh, which is relatively poor in purines, are extraordinarily high, compared with the values for uric acid N in man in corresponding experiments. Burian and Schur [1900] have determined the purine content of different tissues, and they found [1900, p. 309, Table VIII] in fresh horse-flesh 0·065 % purine N, equal to about 2 % of the total nitrogen. It is thus impossible that the purines in 200 g. of horse-flesh can be responsible for more than 0·13 g. purine N, and in 500 g. of horse-flesh for more than 0·32 g. purine N (allantoin N + uric acid N) in the urine.

Most of the excreted allantoin and uric acid must therefore in these experiments have another origin than the purine content of the food. Either the break-down of nucleic materials in the body must have increased when feeding horse-flesh, or purines are synthesised from purine-free protein.

In feeding experiments with 700 g. of horse-flesh on two other dogs the excretion of purine derivatives was less than in the previous experiments with less meat, the excretion being on the average 2·7 and 2·5 % of the total N.

Table II.

Dog C. Female, weight 17·9 *kg.* 700 *g. horse-flesh.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Remarks
12. v. and 13. v.	19·9	198	1·34	66	0·47	Urine put together for 2 days. Average for 24 hours
				2·7 % of total N		

Dog E. Male, weight 19·4 *kg.* 700 *g. horse-flesh.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Remarks
12. v. and 13. v.	20·2	167	1·27	56	0·45	Average for 24 hours
				2·5 % of total N		

Three other dogs were fed exclusively with milk and bread *ad libitum.* They had been on this diet for weeks, and there was no change in the diet when they were placed in the metabolic cage. As appears from Table III there is a difference in the excretion of allantoin and uric acid in these dogs which can neither be due to the difference in the quality of the food, nor to the quantity of protein metabolised. The excretion of allantoin and uric acid is greatest in a dog (dog *G*) which has a smaller excretion of total N than the other dogs. The excretion of purine N is 5·3 % of the total N in this dog, and the total amount is the highest found in any of these experiments. It is evident that the excretion depends upon some other important factor than the existence of certain amino-acids in the proteins supplied.

Table III.

Dog F. Female, weight 14·00 *kg. Bread and milk* ad libitum.

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
1. vi.	22·15	248	1·23	83	0·43
				2·33 % of total N	

Dog G. Male, weight 21·5 *kg. Bread and milk* ad libitum.

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
3. vi.	18·13	436	2·35	145	0·82
				5·3 % of total N	

Dog H. Male, weight 17·00 *kg. Bread and milk* ad libitum.

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.
5. vi.	18·14	157	1·25	52	0·44
				2·7 % of total N	

The suggestion of a synthesis in mammals of purines from purine-free protein dates from recent years. The starting point for all researches on this problem is the observation of Folin [1905] made 20 years ago, that the endogenous uric acid excretion in man is influenced to a considerable extent by the protein content of the food. Folin found that a change from a protein-rich diet, consisting of eggs and milk, to a protein-poor diet, consisting of cream and starch, might be accompanied by a considerable decrease in the excretion of uric acid—a decrease, however, which was not the same in all individuals.

This observation of Folin was later confirmed by a number of investigators [for lit. see Rose, 1921]. Most of the earlier explanations of this phenomenon are based upon the conception that the nucleins are the sole origin of the uric acid in the urine (increased activity of the digestive organs, stimulation of cellular metabolism by amino-acids).

More recently the suggestion was made that the purines might be synthesised from amino-acids. This suggestion is based upon the work of Ackroyd and Hopkins [1915] previously alluded to. These authors found that the allantoin excretion in young rats decreased 40–50 % when the rats were supplied with purine-free diets deprived of arginine and histidine, but complete in every other respect, and that the allantoin excretion again increased when these amino-acids were added to the food. Tryptophan was without any influence on the allantoin excretion. The authors concluded from their experiments that arginine and histidine were usable raw materials for purine synthesis in the body of the rat.

The experiments of Ackroyd and Hopkins are of the greatest importance. They were the first to show that a synthesis of purines from amino-acids occurs in mammals, a synthesis which until that time was known only with certainty in birds and reptiles.

Harding and Young [1919] have thereafter arrived at a similar conclusion as regards dogs. As previously mentioned these authors found that placenta, which is rich in arginine, produced a much greater excretion of allantoin than did meat.

Quite recently Rose and Cook [1925] in experiments on rats have confirmed the results of Ackroyd and Hopkins as far as the relation of histidine to purine metabolism is concerned. On the other hand, arginine failed to affect the output of allantoin.

As regards our experiments, they permit of the conclusion that in dogs on meat diet purine derivatives are excreted in amounts which are extraordinarily high, and which largely exceed the purine content of the meat, and also that dogs on purine-free diet excrete such large amounts of purine derivatives that a synthesis is most plausible.

The experiments do not however permit conclusions as to which amino-acids may be the raw materials for the synthesis of purines.

The most striking feature in our experiments is that the amount of the synthesised allantoin and uric acid varies in the different dogs on the same diet. The power of synthesis is most probably common to all dogs, but the extent of synthesis seems to depend more upon individual factors than on the kind of amino-acids in the food.

That the purine metabolism varies, not only in the different species but also in different individuals of the same species, is a fact. This is evident both from the varying amount of excreted endogenous purine bodies and from the varying power to destroy intravenously injected uric acid, as shown by Folin, Berglund and Derick [1924]. As regards the dog, the velocity with which the intravenously injected uric acid is destroyed, according to these authors, is different in different dogs, but never irregular in the same dog.

When we calculate the ratio allantoin : uric acid in our experiments we arrive at a similar conclusion. There is no constant relationship between these substances in the urines of these dogs. Hunter and Givens [1914] have introduced the term "uricolytic index" to indicate this relationship. The term expresses the percentage of allantoin N on the sum of uric acid N plus allantoin N. In normal dogs Hunter, Givens and Guion [1914] found an index of 98. In studies of the uric acid metabolism in Dalmatian dogs S. R. Benedict [1916, 1917] found that these dogs eliminate more uric acid in proportion to allantoin and that accordingly the index is smaller, varying between 24 and 62.

In other mammals Hunter and co-workers [1914] have found that the index varies between 79 and 98, but is constant in each species, indicating that among mammalia there exist several distinct types of purine metabolism.

The validity of this index as a physiological constant, however, is rendered doubtful, when the "uricolytic index" in our experiments (Table IV) is considered.

Table IV.

Animal (dog)	Uricolytic index (average)	Diet
A	95	200 g. horse-flesh, 100 g. fat
B	91	500 g. horse-flesh
C	87	700 g. horse-flesh
E	89	700 g. horse-flesh
F	84	Bread and milk
G	85	Bread and milk
H	89	Bread and milk

It appears from this table that the "uricolytic index" varies in all these dogs, the highest index being 95, the lowest 84.

It seems therefore as if the oxidation of uric acid also varies individually in the different dogs.

These observations of a varying purine metabolism in animals of the same species support our opinion that the suggested synthesis depends on *individual factors*, which we are inclined to conceive as persisting ontogenetic dispositions.

By studying the comparative biochemistry of the purine metabolism one gets the impression that individual factors play a predominating role.

The varying excretion of "endogenous" uric acid in different human beings, the varying concentrations of uric acid in the blood, the great uric acid excretion in the Dalmatian hound compared with the usual excretion in other dogs, and the varying allantoin and uric acid excretion in different dogs point to individual dispositions, which probably persist from embryonic life and are inheritable, and are such inheritable dispositions as might characterise a family or a race.

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CIX. THE "URICOLYTIC INDEX" IN DIABETIC DOGS.

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The term "uricolytic index" was introduced by Hunter and his co-workers in their extensive investigations of uric acid and allantoin excretion in different animals (literature in the preceding paper). The term expresses the percentage of the total of uric acid N plus allantoin N represented by the allantoin N.

In normal dogs Hunter and Givens calculated an index of 98.

In the preceding paper we have shown that the validity of the "uricolytic index" as a physiological constant is doubtful.

However, assuming that allantoin is the terminal product of purine metabolism in dogs and that the conversion of uric acid into allantoin is due to oxidation, this index still must give information about this oxidation process when feeding or injecting purines.

In two diabetic dogs we have determined this index in feeding experiments with nucleic acids. For control we gave nucleic acid in the same quantities to two normal dogs. The normal dogs were dog *C* and dog *E* in the preceding paper.

Table I.

I. *Dog C. Normal, female, weight* 17·9 *kg.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Uricolytic index	Remarks
12. v. and 13. v.	19·9	198	1·37	66	0·47	87	700 g. horse-flesh Average for 24 hours
14. v. and 15. v.	22·1	291	1·71	97	0·60	87	700 g. horse-flesh 5 g. nucleic acid* Average for 24 hours

II. *Dog E. Normal, male, weight* 19·4 *kg.*

Date	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Uricolytic index	Remarks
12. v. and 13. v.	20·2	167	1·27	56	0·45	89	700 g. horse-flesh Average for 24 hours
14. v. and 15. v.	21·7	229	1·56	76	0·55	88	700 g. horse-flesh 5 g. nucleic acid*

* Merck's commercial preparation.

The experiments show that feeding 5 g. nucleic acid does not alter the uricolytic index in these dogs. The uric acid and allantoin excretion increase proportionally.

The diabetic dogs suffered from a heavy glycosuria. They had a moderate acidosis with increased ammonia production and positive ferric chloride reaction.

They were kept on a diet consisting of 500 g. of horse-flesh and 50 g. emulsified fat. The urine was collected in a metabolic cage during three days. During the three days following the dogs received in addition to the same diet 5 g. nucleic acid per day and thereafter during the next three days the same diet without nucleic acid. The total N, uric acid and allantoin were estimated and the average calculated for 24 hours. The methods used were the same as in the preceding paper. The results appear in Table II.

Table II.

III. *Dog K. Diabetic, weight* 8·00 *kg.*

Period	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Uricolytic index	Remarks
1	14·18	292	1·63	97	0·57	85	500 g. horse-flesh 50 g. fat per day
2	15·04	516	1·99	172	0·67	81	500 g. horse-flesh 50 g. fat, 5 g. nucleic acid
3	14·28	232	1·27	77	0·45	85	500 g. horse-flesh 50 g. fat

IV. *Dog L. Diabetic, weight* 8·70 *kg.*

Period	Total N g.	Uric acid mg.	Allantoin g.	Uric acid N mg.	Allantoin N g.	Uricolytic index	Remarks
1	14·07	131	1·18	43	0·41	90	500 g. horse-flesh 50 g. fat
2	14·91	286	1·33	95	0·46	83	500 g. horse-flesh 50 g. fat 5 g. nucleic acid
3	14·17	254	0·91	84	0·32	79	500 g. horse-flesh 50 g. fat

The findings in these diabetic dogs are different from those in the normal dogs. The "uricolytic index" decreases when feeding nucleic acid, indicating a diminished power to destroy uric acid.

This diminished power to oxidise uric acid must be due either to general diminution of the oxidative power of the body, or to lack of the special uric acid-oxidising agent.

The conception of a general diminution of the oxidation power of the body would be in accordance with the concept of the nature of diabetes as a diminution of the carbohydrate-oxidising processes.

However, Folin, Berglund and Derick [1924] have recently demonstrated that the pancreas has nothing to do with the oxidation of uric acid. Into a dog whose pancreas had been removed about 5 hours before and whose blood-sugar had risen to 0·3 % they injected intravenously 100 mg. of uric acid per kg. The injected uric acid disappeared from the blood just as quickly as in normal dogs.

The disturbance of the carbohydrate metabolism *per se* cannot therefore be the cause of the diminished oxidation of uric acid found in our dogs.

We are inclined to conceive the increase in the uric acid excretion as caused by liver insufficiency. The uric acid-oxidising enzyme, uricase, is probably formed in the liver—the presence of it in the liver is shown by Wiechowski. Moreover, the large increase in the uric acid concentration of the blood after liver extirpation in dogs, as demonstrated by Mann and Magath [1921], also makes it probable that the uric acid-destroying agent is formed in the liver. Pathological conditions, however, arise in the liver during the disease, as shown by the formation of ketones, the lack of glycogen formation and the fatty infiltration of the liver cells.

As a consequence of such an insufficiency the uric acid-destroying agent is probably produced in less amount, or the milieu is not favourable to its action.

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CX. A NOTE ON THE PRESENCE OF GLUTATHIONE IN THE CORPUSCLES OF MAMMALIAN BLOOD.

By HENRY FRANCIS HOLDEN (*Beit Memorial Research Fellow*).

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(*Received July 18th, 1925.*)

The work to be described was undertaken with the intention of investigating the optically active substances of blood other than glucose.

It was stated by Winter and Smith [1922] that the optical rotation of a concentrated protein-free filtrate of mammalian blood was initially less than that calculated from the amount of glucose estimated to be present. This difference they attributed to the presence of "an unstable modification of glucose."

The writer observed that protein-free filtrates of the corpuscles of sheep and ox blood were free from glucose but contained a laevo-rotatory substance in significant amounts and that its rotation decreased with increase in the hydrogen ion concentration of the solution, thus affording a possible explanation of the phenomena recorded by Winter and Smith.

It was decided to attempt to isolate and identify this substance. The process was necessarily tedious since the optical properties threw little light on its chemical nature.

After numerous attempts with a variety of precipitating agents the following method was employed.

Four litres of sheep's blood were freed from protein with tungstic acid [Folin and Wu, 1919], and the precipitate well washed with cold water. To the united filtrate and washings were added 35 cc. of 10 % mercuric sulphate in 5 % sulphuric acid for each litre of fluid. The precipitate was filtered off and well washed with cold water. It was decomposed with hydrogen sulphide and the sulphuric acid exactly removed with baryta. The filtrate was concentrated under diminished pressure to 50 cc. and enough sulphuric acid added to make the solution semi-normal. Phosphotungstic acid dissolved in semi-normal sulphuric acid was added till the maximum precipitate was obtained. The precipitate was filtered off, well washed, and decomposed with baryta: the barium tungstate was removed by filtration and the excess baryta removed with sulphuric acid. The solution was concentrated to a syrup and on addition of alcohol a white powder was precipitated.

The specimen thus obtained proved to contain both "neutral" sulphur and amino nitrogen. After reduction it yielded the nitroprusside reaction. It had the same melting point as glutathione from other sources and when mixed with a specimen of pure glutathione from yeast the melting point was unchanged. After acid hydrolysis characteristic crystals of glutaminic acid hydrochloride were obtained.

No trace could be found of it in sheep's plasma, which is in accordance with Arnold's [1911] and Hopkins' [1921] earlier experiments using the nitroprusside reaction. Hopkins found that phosphotungstic acid precipitates gluthathione if the latter is in a moderately concentrated solution. He accordingly worked at a low concentration, whilst in the present case the solution was previously concentrated in order to assist the precipitation.

Subsequent experiments indicate that it is present in the corpuscles of sheep, goats, rabbits and rats in the reduced form. Its presence was apparently overlooked by Tunnicliffe [1925].

The yield of 0·05 g. per litre obtained is probably considerably below the actual concentration, since the methods of precipitation involve a considerable loss.

It may be of interest to note that both cysteine and reduced glutathione have the power to convert methaemoglobin into oxyhaemoglobin and to reduce the latter to haemoglobin *in vitro*. This may afford an explanation of the rapid conversion of methaemoglobin into oxyhaemoglobin in the animal body.

SUMMARY.

Glutathione has been identified as a constituent of the red corpuscles of sheep's blood.

It apparently exists in the corpuscles of many animals in the reduced form.

It is apparently the chief optically-active constituent of de-proteinised blood other than glucose and as such is responsible for the effects recorded by Winter and Smith and others and quoted as evidence in favour of the presence of an unstable modification of glucose.

It also probably accounts for much of the "neutral" sulphur of blood.

The writer desires to record his gratitude to Sir F. G. Hopkins for his continual interest and advice.

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CXI. A NOTE ON AN IMPROVED TECHNIQUE FOR USE WITH SYNTHETIC DIETS.

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(*Received July 21st, 1925.*)

The usual method of making synthetic diets is to mix all the dry constituents together and then add sufficient liquid to form a stiff dough. It appears to me that there are many drawbacks to this method of procedure. Primarily, such a mixture bears no resemblance at all to the ordinary articles of food. It is much too concentrated and does not contain anything like the quantity of water usually found in foods (the common foods vary from 40–90 % in water content, e.g. meat 40–70 %, bread 40 %, vegetables 76–96 % [Plimmer, 1921]). In consequence the animals are being fed too small a bulk.

About a year ago, I started using synthetic diets, having previously fed rats on different foods using white bread as a basis. I was immediately struck by the difference in general condition of the animals. When on a synthetic diet the rats were limp to feel, whereas the animals fed on ordinary foods were far fitter and their muscles of better tone. Also the rats fed on the synthetic diet became pot-bellied and had flaccid abdominal walls; some died and *post mortem* examination showed a dilated large intestine, frequently with gaseous contents. I understand from other researchers that this condition is usually present in animals fed on synthetic diets and that nutrition workers have attempted to remedy it by adding some form of "roughage," such as agar, filter paper, charcoal, etc. to the diet. Even with such addition I feel that the bulk taken by the animal is far too small as compared with the normal consumption of food. Therefore, I tried to devise some method by which more water could be incorporated in the synthetic food, so that its composition should approximate more closely to that of an ordinary mixed diet. It was impossible to add more water with the present method because on standing the liquid separated out on top. The next attempt was to add more water to the diet and heat in a saucepan, stirring well until the starch grains burst and so thickened the mixture. By this means no water separated out and a diet prepared in this way gave excellent results when fed to rats. Details of the preparation are given below.

EXPERIMENTAL.

(*a*) *Foods.*

The composition of the diet used in these experiments was as follows:

20 g.	caseinogen.
64 g.	starch.
4 g.	salt mixture [Hartwell, 1922].
12 g.	butter.
2 g.	yeast extract (marmite).

The caseinogen, starch and salt mixture were ground together in a mortar in bulk. The butter and marmite were added to the ration daily, so that the food was freshly made. The marmite was used as a 5 % solution. This diet may be adversely criticised as being too low in vitamin B, since the majority of nutrition workers in England use 5 g. marmite in 100 g. synthetic diet. The experiments quoted here form part of a series of experiments now in progress to determine the relative amounts of vitamin B required by the lactating and non-lactating rat, and as yet no data have been given for the minimum vitamin B needs of the rat (except by Osborne and Mendel [1922] who find that at least 200 mg. of compressed yeast per day are necessary for it to attain its full weight). In these experiments the average daily intake per animal was 0·2 g. marmite for a rat weighing about 40 g., whilst with older animals the marmite ration was as much as 0·5 g. per day.

Lemon juice was not added to the diet, because it was desirable that the marmite should be the only source of vitamin B. The antiscorbutic vitamin has been omitted in previous experiments of the author's without any detrimental results [Hartwell, 1924], and Simmonds [1924] states that "the rat is apparently immune to scurvy and does not require this vitamin in its diet."

The two diets were prepared as follows:

Method 1. 22 g. of the dry constituents, 10 cc. of 5 % solution of marmite, 6·5 cc. distilled water and 3 g. butter (melted) were beaten together to form a stiff dough.

Thus the total liquid used with 22 g. solid was 16·5 cc.

Method 2. A double saucepan was used. 22 g. of the dry constituents were mixed to a smooth paste with warm distilled water (about 40°), the 10 cc. of marmite solution and the 3 g. butter (melted) were then added and the whole well mixed. Then more distilled water (40°) was added, the total amount used being 65 cc. to the 22 g. of dry solid. This mixture, in the inner saucepan, was then placed in the outer pan containing boiling water and stirred rapidly until it thickened, after which it was left for 1 minute before being removed from the gas. When made in this way, the synthetic diet resembles a rather firm blancmange. The time required for thickening, 2½–3½ minutes, is too short for any destruction of vitamins to take place. Very hot water cannot be used or the mixture becomes lumpy.

Thus for 22 g. dry food in Method 1, 16·5 cc. liquid were used, whilst in Method 2, 75 cc. were needed. Microscopically this "cooked" food differs from the "raw" mixture in that the starch grains are swollen and the majority are broken.

The animals were not given a definite ration, but were fed so that small quantities were left.

(*b*) *Experiments.*

Exp. 1. *Method of preparing diet changed part way through the experiment.* Twelve rats, 6 ♀ and 6 ♂, weighing about 40 g. each, were given the synthetic diet in the usual form, *i.e.* mixed to a stiff dough. After 19 weeks on this diet the method was changed and the food "cooked" as described above. The experiment was continued for 17 weeks longer.

Exp. 2. *Method of preparing the diet kept constant,* but two groups of animals used, one group being fed on the "raw" and one on the "cooked" food.

A. Rats weighing approximately 40 g. at the beginning of the experiment; 12 animals, 6 ♀ and 6 ♂, fed on each diet.

B. Rats weighing approximately 70 g. at the beginning of the experiment; 8 animals, 4 ♀ and 4 ♂, fed on each diet.

Results.

1. *Growth.* At first the rate of growth of all the rats was equal but after a few weeks the growth curve of those fed on the "cooked" diet was better than that of those fed on the "raw." The average weights of the male rats at the beginning and end of the experiments are given below. (The average weights of the does cannot be given because they became pregnant at different times.)

	Average weight at beginning of experiment	Average weight at end of experiment (10 weeks)	Diet
Exp. 2. A.			
	39 g.	215 g.	"Cooked"
	39	197	"Raw"
Exp. 2. B.			
	69 g.	242 g.	"Cooked"
	68	229	"Raw"

In Exp. 1 the growth curve improved when the "cooked" method was adopted.

2. *General condition of the animals.* The difference in the condition of the two groups of rats was quite obvious. From the first the rats fed on the "cooked" diet were the better animals. They were firm to feel, and equal in all respects (except rate of growth) to the control animals fed on a mixed diet of kitchen scraps, supplemented with bread and milk. The rats fed on the "raw" synthetic diet were limp and pot-bellied. At one period their fur came out, but eventually grew again. This loss of fur (thought to be due to some

dietetic deficiency [Boas, 1924; Hartwell, 1924, 1925]) did not occur at all in any of the rats fed on the diet prepared by Method 2; but every animal on the "raw" diet (three experiments with 32 rats) lost some of its fur and showed bald patches for a week or two. The fact that these differences occurred with diets of the *same* composition, but prepared differently suggests that the "cooked" food can be metabolised more effectively in the animal's body.

3. *Post mortem examinations.* These were made by Miss W. M. Clifford who had no knowledge of the diets used.

The rats fed for the whole experiment on the "cooked" diet were normal when compared with those fed on an ordinary mixed diet, whilst those fed on the "raw" diet had a very enlarged caecum and distended large intestine, with prominent lymphatics.

SUMMARY.

A method is described for the preparation of synthetic diets so that the composition and texture approximate more closely to those of ordinary foods.

Evidence is given that rats fed on a diet prepared by this method grow more quickly and are in better condition than others fed on the same synthetic diet mixed to a stiff paste with water in the usual way.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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CXII. NOTE ON THE EFFECT OF HIGH TEMPERATURES ON THE ACCESSORY FOOD FACTOR CONTENT OF COD-LIVER OIL.

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(*Received July 27th, 1925.*)

The experimental work described in this paper was undertaken because of the result obtained on incidentally testing a sample of heated cod-liver oil for its content of vitamin A. This sample of oil had been used to see whether it contained any volatile constituent up to a temperature of 300° at a pressure of 0·2 mm. The pressure above the oil was reduced to this figure before heat was applied to the surrounding oil bath. Then the temperature of the flask contents was raised slowly to 300°, this occupying a period of 6 hours. The flask contents were immediately cooled, air being excluded. Incidentally this oil was tested on rats, and it was found that even 1 g. added daily to the basal diet failed to show any evidence of the presence of the growth factor. Further, X-ray examination of the bones showed that the calcifying principle had also been destroyed during the process. This experiment indicated that heat alone, apart from any external oxidation, could bring about the destruction of these factors—if distinct. A series of experiments, which will be described later, was therefore carried out to determine the effect of heat alone at a lower level on the vitamin content of cod-liver oil.

The stability of vitamin A to heat and oxidation has been the subject of several investigations. The discrepancies between the results obtained by earlier workers in this field were shown by Hopkins [1920] to be due to the fact that the growth-promoting factor is easily destroyed by the oxygen of the air. He showed, on the other hand, that if butter be heated, air being carefully excluded, its vitamin A content is not much reduced by a temperature of 120° maintained for 12 hours.

More recently the view of E. Mellanby [1918] that the anti-rachitic factor is probably identical with vitamin A, has been attacked on the ground that the two principles are not equally stable when exposed to heat and oxidation, the former factor proving more resistant to these processes [McCollum, Simmonds, Becker, and Shipley, 1922; Goldblatt and Zilva, 1923].

In consequence of these results it was decided to determine the effect of heat alone for varying periods on the vitamin content of cod-liver oil. It has already been stated that the author found that slowly heating cod-liver oil to 300°, air being rigidly excluded, destroyed the growth-promoting and calcifying factors. A lower temperature, 200°, was therefore decided upon. A sample of unrefined cod-liver oil was obtained and 200 g. of this placed in each of three long-neck round-bottomed flasks fitted with a rubber cork having inlet and outlet tubes and a thermometer. The outlet tubes were connected up with tubing, the other end of which dipped under mercury, thus excluding air, but allowing escape through expansion in the flasks. By means of the inlet tubes the flasks were flushed out with dry carbon dioxide before heating was commenced. The flasks were heated in an oil-bath. The temperature of the flask contents was gradually raised to 200°, this process taking about 1 hour. One flask was thereupon disconnected and cooled, air being excluded. This specimen will be referred to as Heated 1 sample. The temperature of the oil in the other two flasks was maintained at 200° for 2 hours, when one of these was disconnected and cooled. The contents of the remaining flask were maintained at 200° for a further 4 hours. The flask was then disconnected and cooled, air being excluded. The last two lots of oil will be referred to as Heated 2 and Heated 3 samples respectively.

Effect on the Growth Curve.

A preliminary testing of these three samples for their content of the growth-promoting factor was then carried out on rats, the basal diet of these animals being: starch 50 %, caseinogen 20 %, oxidised heated olive oil 20 %, marmite 5 %, salt mixture 5 % [McCollum, 1917]. It was found that 5 mg. of the unheated sample restored normal growth when this had ceased on the basal diet. About 40 mg. of the Heated 1 sample, and 180 of the Heated 2 sample were required for this purpose, whilst 0·3 g. of the Heated 3 sample gave a very flat growth curve. It is curious to note that while the rats did not grow much on the last sample, yet in no case did they show xerophthalmia.

In Fig. 1 are illustrated the growth curves of a family of ten rats, pairs of which were being tested preventatively with the same amount (namely, 0·1 g. per rat per day) of the unheated and heated specimens of oil, one pair remaining on the basal diet for control. Similar results were obtained with two other sets of rats.

It will be seen that the growth curve given by 0·1 g. of Heated 1 sample is normal, that the growth produced by the same amount of Heated 2 sample is somewhat below normal, whilst the animals fed with 0·1 g. of Heated 3 specimen put on weight extremely slowly.

Relative calcification effects produced by the samples.

With the diet given, great differences in the calcification of bones were not to be expected. The bones were examined radiographically and their calcium content subsequently estimated. The radiograms of the respective sets showed that the bones of the rats on the Unheated and Heated 1 specimens were well and equally calcified. Those of the rats on the Heated 2 specimen showed some osteoporosis, which was more marked in the case of those on the Heated 3 specimen. The calcification in this last case showed a slight improvement on that in the bones of rats on the basal diet only.

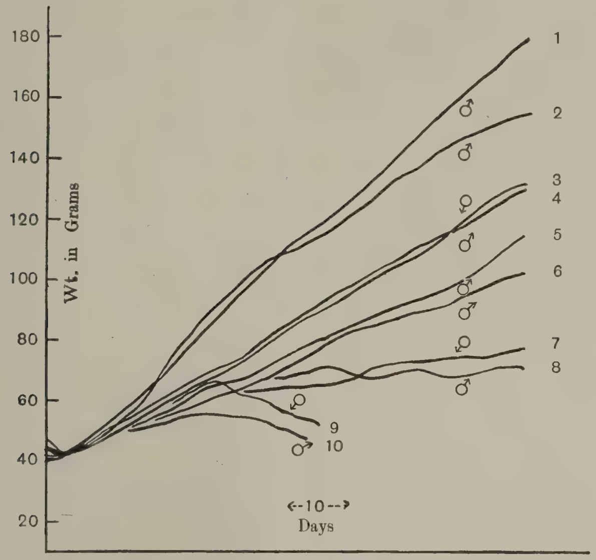

Fig. 1. Rats 1 and 3. 0·1 g. unheated oil
,, 2 ,, 4. 0·1 g. heated 1 sample
,, 5 ,, 6. 0·1 g. ,, 2 ,,
,, 7 ,, 8. 0·1 g. ,, 3 ,,
,, 9 ,, 10. Basal diet only

The calcium content of the femora of the respective animals worked out as follows:

First set of rats

Specimen of oil	% content of moisture	% content of calcium	
		Wet bone	Dry bone
Unheated			
1st rat	25·7	17·0	22·9
2nd rat	23·1	15·9	20·4
Heated 1			
1st rat	22·0	15·1	19·4
2nd rat	23·5	15·0	21·2
Heated 2			
1st rat	28·3	12·6	17·8
2nd rat	29·7	11·9	17·0
Heated 3			
1st rat	31·6	10·3	15·1
2nd rat	31·8	9·0	14·7
Basal diet only			
1st rat	31·2	10·0	15·5
2nd rat	33·6	8·5	12·6

The bones of the other two sets of rats were not analysed for calcium content.

Thus it will be seen that the calcium estimations bear out on the whole the results found radiographically; that is, there is a gradual disappearance of the anti-rachitic vitamin when the oil is heated to, and maintained at, this high temperature.

SUMMARY OF RESULTS.

1. When cod-liver oil is slowly heated out of contact with air to 300° the anti-rachitic and growth-promoting factors contained in it appear to be completely destroyed.

2. If cod-liver oil is slowly heated to 200° and maintained at this temperature for some hours these two factors slowly disappear *pari passu.*

3. Oxidation from external sources is obviously unnecessary for the destruction of these factors at these temperatures.

4. If oxygen be excluded the chemical compound or compounds representing these factors are fairly stable to heat.

The work described above was carried out during the first half of last year, and the main results reported in June, 1924, to the Science Committee of the British Medical Association to whom grateful acknowledgment is made for the grant which defrayed the expenses of the work. Since then Steenbock and his collaborators [1924] have published the results which led them to the conclusion that the presence of the anti-rachitic factor is necessary for the manifestation of growth also.

The possibility of the destruction of the factors in the above experiments being due to the action of the fatty acids liberated by hydrolysis of the glycerides at those high temperatures was one which the author considered and tried to avoid by heating some oil with a weak base out of contact with air. It was found however that as weak a base as magnesium oxide only increased the rate of hydrolysis with much soap formation.

Finally I would express my thanks to Professor Edward Mellanby for his support throughout the work.

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CXIII. THE EFFECT OF ALCOHOL, UNDER VARYING CONDITIONS OF DIET, ON MAN AND ANIMALS, WITH SOME OBSERVATIONS ON THE FATE OF ALCOHOL IN THE BODY.

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(*Received July 27th, 1925.*)

The relationship of the alcohol concentration in the blood of animals to varying conditions of the food content of the alimentary canal was first put on a broad experimental basis by the work of E. Mellanby [1919]. This worker used mainly dogs for his investigations in which the alcohol blood concentration curve was determined for varying conditions of the food and drink of these animals. A year later [1920] he described further results obtained with a human subject. Previous to this Weissenfeld [1898] noted that the changes produced by the ingestion of alcohol in his respiratory and circulatory system were greatest when the beverage was taken on an empty stomach. Voltz, Baudrexel and Dietrich [1912] gave moderate doses of alcohol to men and found that the content of alcohol in the urine was six times greater when the alcohol was taken on an empty stomach than when its ingestion had followed the taking of food. Hanzlik and Collins [1913] studied the absorption of alcohol from the alimentary tract of cats and dogs. Though these investigators were not primarily concerned with the influence of foodstuffs on alcohol absorption, they showed that substances such as olive oil, cholesterol and bile salts exerted a retarding effect on alcohol absorption. Vernon [1919] carried out experiments on the influence of alcohol on manual work and neuro-muscular coordination and concluded that at the height of its influence alcohol is about twice as active in upsetting neuro-muscular coordination when taken on an empty stomach as when taken with food. McDougall and Smith [1920], working independently, corroborated Vernon's results. The extensive results published from the Nutrition Laboratory of the Carnegie Institution of Washington have dealt in the main with the psychological effects of alcohol, but among these records are some which illustrate the effect of food on alcohol ingestion. Thus Miles [1924] showed that when a dose of alcohol was taken shortly after the ingestion of a full meal, the effect, as exhibited in the accuracy and speed of neuromuscular processes involved in typewriting during a 4-hour interval following the ingestion, was very much reduced compared with the effect when no food had been taken. With the exception of Mellanby the above-mentioned authors have not dealt mainly with the effect of foodstuffs *qua* foodstuffs on the alcohol blood concentration. The work to be described is a more detailed study of the relation of alcohol to diet in the human subject. Three men, who were moderate drinkers of alcoholic beverages, were chosen. They were J. H. B. age 53 years, weight 11st. 4 lb., an army pensioner after 23 years in the R.F.A. who continues at his trade as saddler; G. H. N. age 31 years, weight 11 st. 8 lb. and his brother W. N. age 34 years, weight 9 st. 8 lb. were foundrymen, but for the past 18 months have been unemployed. The investigations covered the period July, 1923–December, 1924. During this interval the weight of the three men remained sufficiently constant to need no change in the amount of alcohol given. Not more than one experiment was carried out per week and the routine was always the same. Their last meal on the day preceding that of the experiment was consumed between 8 and 9 p.m. Alcoholic drinks were, of course, not allowed on this day and no liquid of any kind after 9 p.m. No food or drink was taken before arrival at the laboratory on the morning of the experiment. The dose of alcohol was the same for each man and for each experiment, namely, 1200 cc. of (practically) 8 % alcohol made by adding 300 cc. of absolute alcohol to 3450 cc. of tap water, *i.e.* 96 cc. of absolute alcohol per man. This dose produced little or no evidence of intoxication. There was no difference in their ability to draw a simple diagram, to walk or run, an hour after taking the alcohol from that before the beverage was taken. No flavouring was added to the alcohol, the drinking of which was always spread over 10 minutes. The men rested in the laboratory, the temperature of which was maintained as nearly as possible at 17–18°. In those experiments in which food was taken before the alcohol, the interval between the consumption of the food and taking alcohol was always 1½ hours with the single exception of the period (2 hours) after olive oil. The alcohol content of the blood, urine, etc., was recovered by distillation in the apparatus previously described by the author [1924] and estimated by the slightly modified Pringsheim's method described therein. Blood samples (about 10 g.) were taken at the following intervals after the completion of drinking the alcohol, 1, 2½ and 6½ hours. In several of the experiments the concentration of alcohol in the urine was also determined at intervals. Whether the urine was kept for estimation or not, the times for evacuating the bladder were the same in all the experiments, namely, just before the dose of alcohol, then every 20 minutes during the next 2½ hours, then every ½ hour.

In the accompanying table the concentration of alcohol in the blood is given in cmm. of alcohol per 100 g. blood; the concentration in the urine in cmm. of alcohol per 100 cc. urine. In some of the experiments the whole of the urine for the 6½ hours was pooled and the mean figure for the alcohol content also determined.

The experimental results are set out in the following table:

Table I. *The variation of the alcohol concentration in the blood and urine of the resting subject with varying dietetic conditions.*

		Alcohol concentration in the blood after			Alcohol in urine		Alcohol in urine / Alcohol in blood	
Food taken	Subject	1 hr.	2½ hrs.	6½ hrs.	2½ hrs.	6½ hrs.	2½ hrs.	6½ hrs.
(*a*) No food	J. H. B	146	148	87	193	114	1·37	1·37
	G. H. N.	151	148	78	199	103	1·39	1·34
	W. N.	160	154	85	212	126	1·38	1·40
(*b*) 530 cc. water	J. H. B.	142	145	81	205	127	1·41	1·56
	G. H. N.	130	141	74	199	104	1·41	1·40
	W. N.	170	154	92	197	125	1·28	1·36
(*c*) 530 cc. whole milk previously boiled	J. H. B.	114	130	58	186	87	1·43	1·50
	G. H. N.	123	134	62	184	100	1·37	1·40
	W. N.	Lost	141	71	196	101	1·39	1·42
(*d*) 530 cc. whole milk previously boiled and 183 g. bread	J. H. B.	106	116	48	161	70	1·39	1·46
	G. H. N.	125	116	45	162	58	1·40	1·26
	W. N.	128	123	49	180	82	1·46	1·67
(*e*) 530 cc. water and 183 g. bread	J. H. B.	Lost	125	58	163	83	1·30	1·43
	G. H. N.	137	126	62	164	92	1·30	1·48
	W. N.	159	143	70	191	114	1·34	1·63
(*f*) 50 g. separated milk powder, 183 g. bread, 500 cc. water	J. H. B.	101	117	44	146	67	1·60	1·50
	G. H. N.	140	123	52	154	76	1·25	1·46
	W. N.	157	130	52	182	78	1·40	1·50
(*g*) 50 cc. olive oil	J. H. B.	143	142	79	203	114	1·43	1·44
	G. H. N.	126	146	79	217	125	1·49	1·58
	W. N.	126	155	88	226	128	1·46	1·45

The effect of diet on the concentration of alcohol in the blood (Table I).

(*b*) In these experiments there is no increased rate of absorption when water is previously taken. Mellanby found that a previous drink of water increased the absorption rate of alcohol in the case of dogs.

(*c*) The marked effect of a previous draught of milk in lowering the alcohol blood concentration curve, which Mellanby noted in his experiments is well corroborated.

(*d*) Taking a meal of bread and boiled whole milk previously has an even greater effect than milk alone in lowering the concentration curve which is given by alcohol on an empty stomach.

(*e*) Exps. (*c*) and (*e*) show that the meal of bread and water has about an equal effect to that of whole milk on the subsequent blood concentration curve under the above experimental conditions.

(*f*) This experiment was designed to simulate Exp. (*d*), the milk-fat being absent. The figures in these two experiments agree on the whole quite well. Exps. (*e*) and (*f*) suggest that fats are not the only factor tending to prevent alcohol from circulating in the blood stream.

(*g*) This experiment was carried out to see whether fats as such have any specific effect on subsequent alcohol absorption. In the case of J. H. B. it will be seen that there is little difference between the concentration curves of alcohol in the blood, on an empty stomach, after a drink of water, or after taking olive oil. In the case of the other two subjects there is evidence of delayed absorption at first, but subsequently the concentration curves after olive oil practically coincide with the respective curves obtained when the alcohol is taken on an empty stomach. On the whole, therefore, it would seem improbable that it is the fat of milk or of bread and milk, as Mellanby [1920] suggested, which is responsible for the alcohol blood concentration curve being so depressed after these foodstuffs.

It is noteworthy that after the maximum concentration of alcohol in the blood has been reached the rate of fall in the concentration of alcohol in the blood is the same for the same individual in all these cases, provided he be kept at rest, and external conditions, especially temperature, be kept constant. This fact was noted by Mellanby in dogs. The results here suggest that within the limit of experimental error this rate of disappearance is the same for all three men, but further experiments will have to be carried out before this can be regarded as established.

This fact of the rate of disappearance of alcohol being independent of the concentration when once the maximum has been reached raises an important question, namely, the fate of a large fraction of the alcohol taken after a meal of bread and milk. For it is obvious from the table that a large fraction of the ingested alcohol after a previous meal of bread and milk never reaches the blood stream, or, if it does, disappears from this at once. The following attempts, unfortunately unsuccessful, were therefore made to trace the fate of this alcohol:

(1) The first solution that suggested itself was that a considerable fraction of the alcohol had never been absorbed, being perhaps adsorbed or retained in some way by the food. To test this, two dogs were given alcohol, one on an empty stomach, the other when a meal of bread and milk had been given 1½ hours previously. Blood samples were taken at intervals; after 2½ hours the animals were instantaneously killed, and the alcohol in the contents of the alimentary canal estimated. The gut wall was not included in this estimation. The results were as follows:

Dog A

Weight	Food taken	Alcohol given	Alcohol in 100 g. blood after	Total alcohol in
15 lb.	No food after 6 p.m. previous evening or water after 8 p.m.	9·75 cc. diluted to 122 cc.	14 mins. 152 cmm.	Stomach 20 cmm.
			44 ,, 184 ,,	Small intestine 0
			85 ,, 175 ,,	Large intestine 20 cmm.
			134 ,, 161 ,,	
			140 ,, dog killed	
Dog B				
13 lb.	33 g. bread, 250 cc. milk	8·5 cc. diluted to 106 cc.	18 mins. 90 cmm.	Stomach 280 cmm.
			50 ,, 134 ,,	Small intestine 40 cmm.
			116 ,, 131 ,,	Large intestine 110 cmm.
			137 ,, 116 ,,	
			144 ,, dog killed	

An examination of these figures shows that the amount of alcohol in the lumen of the gut of dog A at the time of killing is practically negligible. From dog B 0·43 cc. of alcohol was recovered. A set of experiments was also carried out to see whether known amounts of alcohol added to bread and milk under conditions simulating those existing in the stomach and small intestine, could be recovered. It was found that when 10 cmm. alcohol in dilute aqueous solution were added, on the one hand, to 10 g. bread and milk with pepsin and hydrochloric acid, and on the other to 10 g. bread and milk with pancreatic extract and sodium carbonate, and each sample incubated for 2 hours at 37°, the alcohol could be recovered practically completely from both mixtures. It would appear therefore that the alcohol recovered from the contents of the alimentary canal represents the whole of that compound present. If this can be assumed then it is obvious that the presence of 0·43 cc. of alcohol in the alimentary tract of the second dog will not explain fully its low alcohol blood concentration figures compared with those of the first dog.

(2) The low figures for the alcohol content of the alimentary canal of dog A rather rule out the idea that there is a back secretion of the alcohol into the lumen of the gut. Gréhant [1903] found that when he gave large concentrated doses of alcohol intravenously to dogs there was a secretion of this into the stomach. Hanzlik and Collins [1913] found that when alcohol was placed in the stomach or in isolated loops of intestine there was a re-secretion of this compound into the intestine, though the amount was small. Previous to the experiment with the two dogs the present writer had carried out a series of experiments on rabbits to test the degree of back secretion into the alimentary tract. The animals were given no food for 2 days previous to the experiment and their drinking water was withdrawn on the evening preceding the experiment. The alcohol was given intravenously. The following results were obtained:

Rabbit	Weight	Alcohol given	Interval between the alcohol and killing	Concentration in the blood at death	Alcohol found in the alimentary canal
A	5¼ lb.	5 cc. diluted to 50 cc.	25 mins.	208 cmm.	Stomach 75 cmm. Small intestine 35 cmm. Large intestine 46 cmm.
B	5¼	5 cc. diluted to 50 cc.	4¼ hrs.	72 ,,	Stomach 91 cmm. Small intestine 19 cmm. Large intestine 34·3 cmm.
C	6	—	—	—	Stomach equal to 1·4 cmm. Small intestine 0 Large intestine 1·5 cmm.
D	4¾	4·8 cc. diluted to 48 cc.	8 mins.	258 ,,	Stomach 39 cmm. Small intestine 45 cmm. Large intestine 56 cmm.
E	5¾	5·8 cc. diluted to 58 cc.	11½ hrs.	3 ,,	Stomach equal to 2·8 cmm. Small intestine 0 Large intestine 0
F	4¾	4·8 cc. diluted to 48 cc.	5 hrs.	67 ,,	Stomach 70 cmm. Small intestine 11·7 cmm. Large intestine 44 cmm.
G	5¼	—	—	—	Stomach equal to 2·1 cmm. Small intestine 0 Large intestine 1 cmm.

It is evident that a secretion into the lumen of the gut takes place along its whole length, though not large in amount. It is curious to note here, as in the case of the two dogs, that the small intestine contains least alcohol. This seems to point to one of two possibilities; either the absorption of alcohol is most rapid from the small intestine [cp. Hanzlik and Collins, 1913] or alcohol is being used up in this part of the tract. It will also be noted that in the case of two control rabbits C and G which were not given alcohol, the amount of volatile reducing substance obtained from the contents of their alimentary tract was negligible in spite of fermentation taking place among the contents of the never empty stomach of the fasting rabbit.

(3) The possibility that the disappearance of the alcohol might be due to some interaction or oxidation taking place during the processes of digestion or bacterial action had to be considered and tested. The following experiments were made.

(*a*) To test the effect of enzymes, well-stoppered bottles containing respectively the quantities given below were incubated for 2 hours at 37°, then the volatile contents were distilled, and the reducing substances in the distillates estimated:

Bottle 1. 10 g. bread and milk, pepsin and hydrochloric acid to make 0·2 %.
,, 2. ,, ,, ground up stomach mucosa and HCl.
,, 3. ,, ,, liquor-pancreaticus and dilute sodium carbonate.
,, 4. ,, ,, liquor-pancreaticus and dilute sodium carbonate and ground up small intestine mucosa.

To each of the above bottles 10 cmm. alcohol were added before incubation.

A corresponding set of bottles containing no alcohol was also put up and similarly incubated. The estimations showed that the alcohol had been recovered practically quantitatively from the contents of bottles 1–4 and that the controls contained none.

(*b*) No organism has been obtained from human faeces which could be classed with the acetic acid producing group. On the other hand the production of alcohol from carbohydrates by members of the *B. coli* group, and by *B. welchii* is a well-known fact. The following experimental evidence shows that when dilute alcohol is added to a suspension of faeces and the mixture maintained at body temperature there is a slow disappearance of the alcohol. 100 g. of freshly passed faeces were emulsified with 150 cc. water and then used in quantities of 10 cc. as follows:

Bottle 1. 10 cc. faeces emulsion + 10 cc. water.
,, 2. 10 cc. faeces emulsion + 5 cc. water + 5 cc. 0·4 % aqueous alcohol.
,, 3. Repetition of 2.
,, 4. Repetition of 2.
,, 5. 10 cc. autoclaved emulsion + 10 cc. sterile water.
,, 6. 10 cc. autoclaved emulsion + 5 cc. sterile water + 5 cc. 0·4 % aqueous alcohol.
,, 7. 10 cc. filtered faeces emulsion and 10 cc. sterile water.
,, 8. 10 cc. filtered faeces emulsion and 5 cc. sterile water + 5 cc. 0·4 % aqueous alcohol.
,, 9. 10 cc. autoclaved filtered faeces emulsion + 5 cc. sterile water + 5 cc. 0·4 % alcohol.

The emulsion of faeces used in bottles 5 and 6 was 0·4 % autoclaved for 20 minutes at 125°. The emulsion of faeces used in bottles 7, 8 and 9 was filtered through an "Imperator" candle. This filtrate was found to be sterile as regards ordinary bacteria. The well-stoppered bottles were then incubated at 37° for 4 hours with the exception of bottles 2 and 3 which were withdrawn after 1 and 2 hours respectively. After incubation a little milk of lime was added to each bottle and the contents distilled. The alcohol discovered or produced was then estimated with the following results:

Bottle 1. Contained no volatile reducing substances.
,, 2. 1·4 cmm. (about $\frac{1}{14}$th) of the alcohol added could not be recovered.
,, 3. 2·7 cmm. (about $\frac{1}{7}$th) of the alcohol added could not be recovered.
,, 4. 5·0 cmm. (about $\frac{1}{4}$th) of the alcohol added could not be recovered.
,, 5. Contained no volatile reducing substances.
,, 6. Practically the whole of the added alcohol was recovered.
,, 7. Contained no volatile reducing substances.
,, 8. 2·6 cmm. (about $\frac{1}{8}$th) of the added alcohol could not be recovered.
,, 9. Practically the whole of the added alcohol was recovered.

There is a definite though small disappearance of the alcohol added to faeces *in vitro*—whether this process is increased or not in the colon cannot be determined. The result obtained with the contents of bottle 8 suggests that the active agent is either a filter passer or else a ferment. At the same time the possibility of the effect being due to adsorption cannot be excluded by the results obtained above, for the process of autoclaving markedly alters the physical properties of the emulsion. Thus while this evidence of the using up of alcohol by the faeces could only account for the fate of a small fraction of the alcohol ingested, it gives some explanation of the fact that alcohol cannot be found in faeces when a dose has previously been taken [Atwater and Benedict, 1902; Pringsheim, 1908].

The effect of diet on the concentration of alcohol in the urine (Table I).

It will be seen that these results for the urine corroborate and extend the work of Miles [1922], who showed that when 27·5 g. of alcohol, diluted to one litre, were given to human subjects it produced an alcohol concentration in the urine 40–50 % higher than that in the blood during the period 40 minutes to 2 hours after the ingestion of the alcohol. In the present experiments in which 96 cc. alcohol diluted to 1200 cc. were given, a similar increase in the concentration of the urine-alcohol above that of the blood was found at the 2½ and 6½ hour intervals after the ingestion of the alcohol. Some further determinations, not given here, gave a similar figure at the 1 hour interval. It will be seen from the table that the ratios for the 6½ hour intervals are on the whole slightly higher than those for the 2½ hour intervals, though in some of the individual experiments the ratios at these two intervals agree quite well. In the case where bread and water were taken previously to the alcohol (Exp. *e*) the ratios at the longer interval are distinctly higher. It is difficult to suggest a cause for this last result apart from some specific dietary effect.

The main fact which emerges from these results is that the ratio of the alcohol concentration in the urine to that in the blood keeps fairly constant over a considerable period of time after taking the alcohol and represents a value for the urine 40–50 % above that in the blood. The work of Miles showed that the contention of Widmark [1916] and Ambard [1920], that the concentration of alcohol in the urine was identical with that in the blood, only held for the first half hour after ingestion, but that afterwards, from 40 minutes to 2 hours, the urine concentration was 30–50 % above that in the blood. The present work shows that this figure practically holds for the subsequent 4 hours as well, and with a much larger dose of alcohol and in greater concentration.

The importance of this fact is urged in view of the present need for a more reliable test for drunkenness. From the above results, as well as those of Miles, it would appear that a close approximation to the alcohol concentration in the blood can be obtained from a knowledge of the actual figure for the concentration in the urine. The alcohol concentration in the blood probably gives us the best means of gauging the intoxication of the subject. Widmark [1915] reported the results of examining the urine of 27 individuals arrested for alcoholic intoxication. The results varied from 450 to below 200 mg. of alcohol per 100 cc. of urine. Not all those arrested were suffering from gross intoxication. In the above experiments the figures for the urine concentration for the 2½-hour period varied from 150–225 cmm. per 100 cc. of urine, and there were no signs of intoxication. Some specimens of urine from persons arrested and subsequently convicted for drunkenness in this city gave figures 360–366 cmm. of alcohol per 100 cc. of urine. Further specimens are being examined in the hope of establishing a figure for the concentration which may be of service in estimating intoxication.

SUMMARY.

1. The effect of certain foodstuffs taken previously to alcohol on the subsequent alcohol blood concentration curve in man is shown.

2. Once the maximum concentration of alcohol in the blood has been reached, provided the subject be at rest and external conditions are maintained constant, then the subsequent rate of disappearance of alcohol from the blood is independent of the concentration.

3. The effect of a previous meal of a foodstuff such as bread and milk in depressing the concentration curve is demonstrated. This is shown not to be due to delayed absorption, but to the fact that a considerable fraction of the alcohol ingested is never manifested in the blood stream.

4. Experiments are recorded of the attempts made to trace the fate of this fraction of the alcohol which "disappears."

5. The concentration of alcohol in the urine exceeds that of the blood over a period of 1–6 hours after ingestion by about 40–50 %; the passage of alcohol through the kidney into the urine cannot, therefore, be merely a diffusion process.

6. Under the experimental conditions employed the amount of alcohol excreted in the urine varies from 3 to 5 % of that ingested.

In conclusion I would express my thanks to Professor Edward Mellanby for his support and criticisms throughout the work.

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CXIV. A METHOD FOR THE ESTIMATION OF GLUCOSE IN BLOOD.

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So many excellent methods are available for the estimation of glucose in blood, that the introduction of another one requires some justification. The following method probably yields less precise results than several others; but has the advantage of simplicity, and also enables one to determine the approximate amount of sugar in blood without the aid of a colorimeter.

The method is based on the fact that nitro-anthraquinonesulphonates are reduced, when heated with glucose in alkaline solution, first to the corresponding hydroxylamine derivatives having an intense green colour [Wacker, 1902] and later to a deep red substance, which is probably an amine derivative.

The red-coloured final product of reduction is only sparingly soluble in water, and consequently the method is only applicable to dilute solutions of glucose.

A number of nitro-anthraquinonesulphonic acids are known, the chief of these being the 1·6-, 1·7-, α- and β-acids of Claus [1882] and the 1·5- and 1·8-acids [Schmidt, 1904]. These acids, especially the 1·6-acid, are decomposed more or less readily when heated with caustic alkalies, and more slowly by alkaline carbonates, the NO_2 radical being replaced by OH [Frobenius and Hepp, 1907]. The 1·5-acid was found to be the most resistant to the action of alkali, and was therefore selected for the following method. It can be easily prepared in the way described by Schmidt. The isolation of the compound is greatly facilitated by filtering it off through the fritted glass filter discs devised by Prausnitz. Filtration through asbestos is a much slower process. The crystalline precipitate thus separated was purified by recrystallisation from water, and dried *in vacuo* at room temperature.

All the nitro-anthraquinonesulphonic acids have the power of precipitating proteins, the most effective in this respect being the 1·6-acid, but the nitro-anthraquinonesulphonic acid cannot be used both as a protein precipitant and as a reagent for the reduction colour test, since its concentration must be the same in the standard glucose solutions and in the blood filtrates.

Of the different nitro-anthraquinonesulphonic acids, the 1·6-acid was found to be the most delicate with respect to the glucose reaction, but the 1·5-acid is to be preferred for quantitative work for the reason already given. 0·1 cc. of 0·01 % glucose, 0·1 cc. of 0·4 % solution of the 1·5-acid, and 1·0 cc. of 50 % potassium carbonate solution were made up to 5 cc. and heated in the water-bath for 8 minutes, a similar blank experiment with the reagents being carried out concurrently. Under these conditions a faint red tint was just perceptible in the glucose test when compared with the control.

In view of the fact that blood contains organic substances other than glucose, such as uric acid and creatinine, which act as reducing agents in alkaline solution, it was important to ascertain whether these substances reduce nitro-anthraquinonesulphonates under the conditions of the method for the estimation of glucose to be given later. The ratio of the reducing power of glucose to that of uric acid, dissolved as lithium urate, was found to be 2·35 : 1. The reducing power of creatinine was only about one-tenth that of glucose. These statements only hold when alkaline carbonates are used. In the presence of caustic alkalies the reducing powers of creatinine and urate are increased. The amounts of uric acid and creatinine found in blood cannot therefore seriously affect the calculated glucose values. When the percentages of uric acid and creatinine in blood are much higher than normal, the glucose values may be corrected on the basis of the foregoing ratios.

An intense green colour which later passes into a deep red is obtained when normal urine is rendered alkaline with sodium or potassium carbonate and heated with a solution of the 1·5-acid.

Technique of method.

The proteins are precipitated from blood by the tungstic acid method [Folin and Wu, 1918], the blood being therefore diluted 1 in 10. The mixture of diluted blood with sodium tungstate and $2N/3$ H_2SO_4 is allowed to stand for 30 minutes prior to filtration. 5 cc. of the protein-free blood filtrate are measured into a graduated test tube of 15 cc. capacity. 1·5, 1·8, 2·1, 2·5 and 3·0 cc. of 0·02 % glucose are then measured with a normal pipette or micro-burette of 5 cc. capacity, graduated in 0·02 cc., into five numbered graduated test tubes each of 15 cc. capacity and 12 mm. bore. These quantities are in a geometrical series having an approximate common ratio of 1·2 and correspond to 0·06, 0·072, 0·084, 0·1 and 0·12 % glucose in the undiluted blood with the protein-free filtrate of which they may be found to yield a colour match. If necessary the series may be extended in either direction; but, owing to the sparing solubility of the red product of reduction, the method in this form is only applicable to samples of blood containing not more than 0·16 % glucose. In cases of hyperglycaemia it is therefore necessary to use a smaller quantity of blood filtrate, *e.g.* 2·5 cc., and to make allowance for this fact in the calculation.

One cc. of 0·4 % aqueous solution of 1·5-nitro-anthraquinonesulphonic acid, and 2 cc. of a solution of potassium carbonate, containing 50 g. per 100 cc. of solution, are then added to the contents of each test-tube. All the solutions are then diluted up to 10 cc. with distilled water, and mixed thoroughly. At this stage a small quantity of the sparingly soluble potassium salt of the 1·5-acid may separate as a crystalline precipitate if the temperature of the room be low. The test tubes are immersed in boiling water for 8 minutes, then cooled thoroughly in running water, and the volume of each solution made up to 12·5 cc. In many estimations the volume was simply restored to 10 cc. The red tint of the tube containing blood filtrate is then compared with the colours yielded by the glucose solutions. An approximate estimate of the percentage of blood sugar is thus obtained.

1 cc. of 0·4 % solution of 1·5-nitro-anthraquinonesulphonic acid was found to be sufficient for amounts of glucose up to at least 5 cc. of a 0·02 % solution.

The method can be rendered more accurate by using amounts of standard glucose solution in a geometrical series having a smaller common ratio, *e.g.* 1·1. This procedure was adopted after an approximate result had been obtained by the first method. As a rule only one intermediate glucose standard requires to be prepared.

As regards the degree of accuracy of the method one may infer from the limits of sensitiveness of the colour reaction, on which the method is based, that the minimal error in the estimation of blood sugar in a sample of blood containing 0·1 % glucose is about 4 %. The percentage error would be proportionally greater than this for samples of blood containing less than 0·1 % and less for those containing more than this amount.

If a colorimeter be available, the solution derived from the blood filtrate is finally compared in it with the glucose solution which it most nearly matches. As a rule, the results were found to be most accurate when a slightly higher glucose standard was taken for comparison.

It is important to avoid delay in making the colour comparison, especially when dealing with samples of blood containing as much as 0·16 % glucose, since part of the sparingly soluble red product of reduction gradually separates when the solutions are allowed to stand for several hours.

The sugar was estimated in a large number of samples of blood from normal animals and from animals under the influence of drugs or subjected to operative interference. A few of these results are given in the following table. For purposes of comparison the sugar in each sample of blood was also determined by Folin and Wu's method [1920].

Table I.

	% of glucose		
Source of blood	(new method)	(Folin and Wu's method)	Condition of animal
Rabbit	0·178	0·170	Ether anaesthesia
,,	0·334	0·330	Ether and cooling of pancreas
Dog	0·134	0·121	Ether anaesthesia
Cat	0·43	0·40	Ether and adrenaline
Man (normal)	0·084	0·076	After 4 hours' fast
Ox	0·102	0·096	Normal
Cat	0·03	0·026	During convulsions after insulin.

As will be seen from the table the maximal difference between the two methods is about 10 % except in cases of hypoglycaemia, in which the discrepancy is greater.

SUMMARY.

1. A colorimetric method for the estimation of glucose in blood is described.
2. The method gives somewhat higher values for glucose than that of Folin and Wu.
3. At least 1 cc. of blood is required for an estimation.

I wish to express my great indebtedness to Dr A. E. Campbell and Dr J. S. Loughridge for carrying out a large number of analyses.

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CXV. SOME METABOLIC DIFFERENCES, FOLLOWING X-RADIATION, BETWEEN NORMAL RATS AND RATS IMMUNE TO JENSEN'S RAT SARCOMA.

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When rats are inoculated with Jensen's rat sarcoma, it often occurs that some of them fail to grow a tumour. If subsequently tested by re-inoculation from time to time, they are found to be always resistant and are spoken of as "immune" animals. Certain biological differences have been noted between these "immune" animals and normal animals, but up to the present no definite chemical difference has been demonstrated. It is not proposed in this preliminary communication to deal with the literature of the subject.

Since the response to X-radiation of normal animals and animals inoculated with sarcoma had not been worked out, it was thought that an investigation on these lines might yield interesting results.

Numerous analyses on the blood from these two types of animals, X-radiated and not X-radiated, were made for the following constituents: non-protein nitrogen, urea, creatinine, uric acid, amino-acid nitrogen and sugar. All the determinations were made by the colorimetric methods introduced by Folin.

In the first experiments the whole of the animal was exposed to the X-radiation. A galvanometric method of measuring the dose was employed, but it will suffice now to say that about an erythema dose was given in each case, the spark gap being 18 cm. and the screen being $\frac{1}{10}$ mm. aluminium and 3 mm. wood. The animals were killed at intervals of from 1 hour to 9 days after exposure, and the blood collected from the inferior vena cava into pipettes containing weighed quantities of powdered sodium citrate sufficient to produce 3 % citrate in the blood.

Tables I and II give the results obtained using normal animals. All the results are expressed as mg. per 100 cc. of blood.

An examination of these results shows that X-rays produce no effect upon the non-protein nitrogen and urea content of the blood of normal animals.

Table I. *Normal animals not X-rayed.*

	Non-protein N	Urea		Non-protein N	Urea
1.	54·0	—	7.	61·2	41·5
2.	55·9	—	8.	44·4	32·4
3.	57·4	—	9.	31·0	23·0
4.	61·5	—	10.	40·5	30·0
5.	60·0	33·5	11.	40·0	23·1
6.	58·9	34·2	12.	37·5	24·7

Table II. *Normal animals X-rayed.*

	Time X-rayed	Mean galvo. reading in cm.	Killed after exposure	Non-protein N	Urea
1.	40 mins.	8·49	7 hours	57·0	37·5
2.	40 ,,	8·49	7 ,,	47·6	35·1
3.	40 ,,	10·72	31 ,,	43·8	24·1
4.	40 ,,	10·72	31 ,,	49·2	35·5
5.	30 ,,	5·46	9 days	61·8	31·3

Note. With the galvanometer reading 4·2 cm. an exposure of 1·2 hours gives a dose of one rad, which is the exposure required to kill Jensen's rat sarcoma *in vitro*.

Tables III and IV show the effect of radiation on the blood-constituents of the “immune” type of animal, *i.e.* animals previously inoculated with Jensen's rat sarcoma, in which no tumour resulted. The figures for uric acid, creatinine, sugar and amino-acid nitrogen content are not given, since no change was observed on irradiating normal and “immune” rats.

It will be observed that there is a reduction in the blood-urea content of these “immune” animals and that in many cases the non-protein nitrogen content is also slightly reduced.

Table III. *“Immune” animals not X-rayed.*

	Interval between inoculation and killing	Non-protein N	Urea
1.	12 days	60·7	33·8
2.	18 ,,	63·1	47·4
3.	—	57·2	41·2
4.	—	44·2	36·7
5.	41 days	45·0	—
6.	41 ,,	60·0	—

Table IV. *“Immune” animals X-rayed.*

	Interval between inoculation and killing	Time X-rayed	Galvo. reading in cm.	Killed after experiment	Non-protein N	Urea
1.	35 days	35 mins.	9·28	1 hour	40·0	14·0
2.	35 ,,	35 ,,	9·28	1 ,,	43·0	12·9
3.	29 ,,	40 ,,	10·4	24 hours	48·3	8·5
4.	29 ,,	40 ,,	10·4	24 ,,	45·1	9·0
5.	32 ,,	30 ,,	8·87	3 days	57·2	27·7
6.	32 ,,	30 ,,	8·87	3 ,,	52·8	14·4
7.	32 ,,	30 ,,	5·1	3 ,,	44·9	23·6
8.	32 ,,	30 ,,	5·1	3 ,,	—	15·1
9.	44 ,,	30 ,,	6·8	3 ,,	32·2	9·6
10.	44 ,,	30 ,,	6·8	3 ,,	33·7	11·0
11.	44 ,,	30 ,,	6·8	3 ,,	35·7	10·4
12.	44 ,,	30 ,,	5·8	3 ,,	39·7	12·2
13.	44 ,,	30 ,,	5·8	3 ,,	42·8	12·5
14.	44 ,,	30 ,,	5·8	3 ,,	34·1	14·5
15.	57 ,,	30 ,,	5·46	9 ,,	57·2	32·1

On comparing Tables I and III it is seen that "immune" animals do not differ from normal animals in the non-protein nitrogen or urea blood content prior to X-radiation. A difference is, however, to be observed when these two kinds of animals are X-rayed (cp. Tables II and IV). In the "immune" animals there is a marked reduction in the blood-urea content and in many cases a slight reduction in the non-protein nitrogen content, whereas normal animals do not exhibit these changes after similar X-radiation.

These results suggested an inquiry into the behaviour of tumour-bearing animals under similar treatment.

Table V summarises these results.

Table V.

	Urea mg. %
1. Animal with growing tumour not X-rayed	32·7
2. Animal with growing tumour not X-rayed	45·4
3. Animal with growing tumour X-rayed	44·4
4. Animal with growing tumour X-rayed	43·5
5. Animal with disappearing tumour not X-rayed	31·9
6. Animal with disappearing tumour X-rayed	29·1
7. Animal with disappearing tumour X-rayed	64·4

It would appear therefore that tumour-bearing animals behave in the same manner as normal animals.

In all these experiments the whole animal was exposed to the rays. It was thought that the screening of certain organs might lead to information as to the cause of the fall in blood-urea content in "immune" animals, but the experiments showed that there is no particular organ which is especially acted on by the radiation so as to result in a reduced blood-urea content.

SUMMARY.

No difference in metabolism has hitherto been described between normal animals and animals immune to Jensen's rat sarcoma.

The observations described show that there must be some peculiarity in the metabolism of the "immune" animals, since an exposure to X-radiation causes an immediate fall in the blood-urea content, from a normal value of about 35 mg. per 100 cc., to about 14 mg. per 100 cc. This reaction to X-radiation appears to be a permanent property of "immune" animals.

At the present stage, it would be unwise to offer any explanation of these results, but it is hoped to extend the observations to animals rendered immune by artificial means.

CXVI. A DELICATE COLOUR REACTION FOR THE PRESENCE OF VITAMIN A.

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In a previous communication [1920] we stated that the well-known colour reaction of cod-liver oil with sulphuric acid is given not only by the liver oil of this fish but by the liver fats of all animals, and suggested a relationship of the chromogenic substance to vitamin A. The close parallel between the growth-promoting factor and the sulphuric acid test was afterwards emphasised by Drummond and Watson [1922] and further experience, extending over three years, has fully convinced us that this association is more than accidental. The transient nature of the reaction unfortunately prevents its use for a quantitative colorimetric comparison of different oils and all attempts to stabilise the reaction were unsuccessful.

In the course of work on the activation of cholesterol by ultra-violet light [Drummond, Rosenheim and Coward, 1925], we found that arsenic chloride (and various other reagents, see below) gives with cod-liver oil a brilliant ultramarine blue colour reaction. Further study of this reaction led us to the conclusion that, like the sulphuric acid test, it is characteristic for vitamin A. It has the advantage that the colour persists sufficiently long to allow a colorimetric comparison with a suitable standard.

The reaction is carried out by adding 1 cc. of pure arsenic chloride to one drop of cod-liver oil, and shaking the test-tube at once. The oil dissolves immediately to form a clear blue solution, which in the course of a few seconds assumes a purple tint and gradually fades. The reaction is characterised by a well-defined absorption band extending from λ 550–590. Under the above conditions the band persists for about 5 minutes.

Sensitiveness. The reaction is extremely sensitive. A 1 % solution in light petroleum of a highly growth-promoting Newfoundland cod-liver oil was still found to react intensely with 1 cc. $AsCl_3$ in amounts of 0·05 cc. = 0·5 mg. of oil. The limit was reached with 5 mm.^{3} = 0·05 mg., measured with a Wright's capillary pipette. Compared with the ordinary sulphuric acid test in the same solution the sensitiveness was 20 times as great.

The oil was saponified and the unsaponifiable portion freed from cholesterol by means of digitonin. The cholesterol-free fraction now gave the reaction in a dilution of 1 : 2,000,000.

Relation to vitamin A. The fact that the chromogenic substance resists saponification and is concentrated in the cholesterol-free unsaponifiable fraction of cod-liver oil strongly suggests its close relationship to vitamin A, since it has previously been shown by Drummond and Coward [1922] that this fraction contains the fat-soluble vitamin A. This can be distilled with superheated steam in a nitrogen atmosphere, or in a high vacuum, without losing its activity, and still gives, as we found, the colour reaction with $AsCl_3$ in undiminished intensity. On the other hand, the chromogenic substance, and with it the growth-promoting power, is gradually destroyed by oxidation when a current of air is passed through the oil at 100°: after 30 minutes' aeration the reaction is diminished and is no longer given after 60 minutes.

We have further tested a series of over thirty oils and fats and have found complete agreement between the colour intensity and the growth-promoting activity, as tested by animal experiment. Amongst these samples was the butter made from the milk of a cow which had been fed with cod-liver oil. This butter gave the reaction most intensely as compared with ordinary butters, whilst vegetable oils free from vitamin A gave no reaction. Details of this work will be given in a later communication, together with the results of further tests still in progress.

It is interesting to note that this reaction makes it possible to differentiate the growth-promoting vitamin A from the anti-rachitic vitamin D. We have found that cholesterol which had become highly anti-rachitic by irradiation with ultra-violet light [Rosenheim and Webster, 1925] does not react in the way described above with arsenic chloride[1]. We intend to examine in this direction the oils from the marine diatom, Nitzschia, and of plankton, which were found not to respond to the sulphuric acid test by Drummond and Watson [1922], since in the light of more recent experience it is possible that such growth-promoting power as they exhibit may be due to their content of anti-rachitic vitamin and not to the true, growth-promoting, vitamin A.

Experiments which we made on the diffusibility of the fat-soluble vitamins make it highly probable that they both dialyse through a rubber membrane into light petroleum. The oil recovered from the dialysate gives the colour reaction intensely and is also anti-rachitic, as tested by the animal experiment (unpublished experiments by Rosenheim and Webster).

Nature of chromogenic substance. In searching for an explanation of the chemical nature of the reaction, we found that cod-liver oil (and other liver fats) reacted not only with arsenic chloride, but yielded precisely similar blue colour reactions with a number of heterogeneous reagents, which are known to share with arsenic chloride the property of giving a red colour with cholesterol. These reagents are dimethyl sulphate, trichloroacetic acid, acetyl chloride and benzoyl chloride (the last two only in the presence of zinc chloride). Whilst these substances react with cholesterol only when heated, and yield a permanent red colour (absorption band in green), they react with cod-liver oil at room temperature, the blue colour produced (absorption band in yellow) fading within 5–10 minutes.

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[1] The colour reactions of irradiated cholesterol will be described together with its anti-rachitic power in another communication.

The reaction with trichloroacetic acid is particularly striking and may be carried out either by allowing a drop of cod-liver oil to fall on a few crystals of the acid or by adding 1 cc. of a saturated chloroform solution of trichloroacetic acid to one drop of the oil[1]. The colour produced is slightly more purple than that with $AsCl_3$ and the absorption band is correspondingly shifted to the red. The colour is discharged by alcohol, ether, ethyl acetate, acetic anhydride, glacial acetic acid and 90 % formic acid, but not by benzene, toluene, light petroleum and chloroform. Pure mono- and dichloroacetic acids do not react.

It would be premature to assume from the analogy of these reactions to those of cholesterol that vitamin A is a sterol derivative. The interesting suggestion of Harden and Robison [1923] with regard to the mechanism of the sulphuric acid test and the observations of Whitby [1923] on cholesterol reactions in general, would tempt one to assume as an explanation of the new reactions that they are due to the presence of an aldehydic coupling substance or to a substance allied to cholesterol, possessing an aldehydic group in its molecule. It has indeed been stated by Takahashi [1922] that a fraction of the unsaponifiable matter of cod-liver oil, claimed by him to be vitamin A, possesses aldehydic character, as evidenced by the reduction of Fehling's solution and ammoniacal silver nitrate. We also have found clear evidence for the presence of a reducing substance in cod-liver oil, in so far as it gives Schiff's reaction and reduces phosphomolybdic acid. The reducing substance is destroyed by aeration, but withstands saponification to a considerable extent, and is therefore not likely to be aldehydic. A colorimetric comparison by means of Welmans' [1892] phosphomolybdic acid test moreover convinced us that the amount of the reducing substance in various oils does not run parallel to their vitamin A content, as measured by the animal experiment and by the arsenic chloride reaction. The nature of the reducing substance remains to be investigated.

Another observation we made links the chromogenic substance to the lipochromes, of which carotene especially yields, in light petroleum or chloroform solution, a slate-blue colour with arsenic chloride (and the other reagents). This reaction is distinguished from that of cod-liver oil by being permanent.

In view of the possible relationship between sterols and lipochromes and their general association with vitamin A in plant tissues [Coward, 1923], it is suggested that the arsenic chloride reaction is concerned with a substance derived from these types of synthetic plant products under the influence of sunlight.

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[1] The chloroform solution should be freshly prepared, as it slowly generates phosgene on keeping in light. A light petroleum solution may be used.

Colorimetric estimation of vitamin A. The application of the colour reaction to the evaluation of the vitamin A content of cod-liver oil is still under investigation. Should further work confirm the results, which show, so far in every case examined, that the colour intensity is proportional to the growth-promoting power, it will be feasible to replace the animal experiment to a large extent by a chemical test. Preliminary experiments indicate that even a rough colorimetric comparison with a suitable standard yields results of a higher degree of accuracy than those obtainable by a laborious animal experiment. The presence of large amounts of yellow pigment naturally influences the tint of the reaction, but since modern medicinal oils, the main source of vitamin A, are usually only slightly pigmented there is no difficulty in this respect.

We have so far made use of the arsenic chloride and the trichloroacetic acid reaction, and have taken as our standard of vitamin A a highly active Newfoundland oil. A suitable colour standard for the arsenic chloride reaction is a mixture of 100 cc. crystal violet solution (1 : 10,000) with 50 cc. methylene blue solution of the same strength (both in alcohol). Under the conditions chosen we found that the colour produced by 20 mg. of the oil (= 1 drop from a pointed glass rod of 3 mm. diameter) + 1 cc. $AsCl_3$ matches the standard dye solution diluted in the proportion 3 : 2.

It is advisable to add the reagent by means of a teat-pipette, holding 1 cc., and to make the comparison rapidly within a few seconds after the addition of the reagent. After preparing a set of suitably diluted standard solutions in test-tubes of uniform diameter, we found, after a little experience, no difficulty in ranging all the oils so far examined in a series which agreed with their growth-promoting activity.

One of us (O. R.) is greatly indebted to the Medical Research Council for their permission to work at the National Institute for Medical Research, and to Dr Dale and Dr Dudley for placing the facilities of their department at his disposal.

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CXVII. THE RELATION BETWEEN THE PANCREAS AND THE CARBOHYDRATE METABOLISM OF MUSCLE.

II. ANTIGLYOXALASE AND GLYOXALASE.

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(*Received August 3rd, 1925.*)

1. The lactic acid inhibitor and antiglyoxalase.

In a recent paper on the pancreatic control of carbohydrate metabolism in muscle [Foster and Woodrow, 1924] there was described a factor found in the pancreas and capable of inhibiting the production of lactic acid by chopped muscle *in vitro*. It was pointed out that another substance found in the pancreas, namely the antiglyoxalase described by Dakin and Dudley [1913, 4], might have the same effect. According to these authors antiglyoxalase inhibits the action of glyoxalase, thereby preventing or diminishing the production of lactic acid from methylglyoxal and of mandelic acid from phenylglyoxal. If the lactic acid produced by muscle *in vitro* is derived from methylglyoxal by the action of glyoxalase, its formation would similarly be prevented by the addition of antiglyoxalase to the mixture. If, on the other hand, the production of lactic acid from carbohydrate in muscle is not through methylglyoxal, but some other intermediary product, glyoxalase will not form part of the system and therefore antiglyoxalase will not inhibit its production. It is also true that if a certain factor inhibits the production of lactic acid in muscle, but not the production of α-hydroxy-acids from α-ketonic aldehydes, the lactic acid is probably not derived from methylglyoxal but from some other intermediary compound.

The experiments described below were undertaken with the object of deciding whether the factor which inhibits lactic acid formation is identical or not with antiglyoxalase, and hence to get some evidence as to whether or not methylglyoxal is an intermediary product in the carbohydrate metabolism of muscle.

The presence of methylglyoxal has never been demonstrated in the tissues, and its properties render it extremely unlikely that it could persist in sufficient quantities for this to be possible; but there is every reason to believe that, in the liver at any rate, it can form a stage in the breakdown of glucose. This follows from the work of Dakin and Dudley [1913, 2] on the presence of glyoxalase in the liver and more especially from their perfusion experiments [1914]. There is, however, not the same evidence for its presence in muscle. This will be discussed more in detail in Part III of this paper.

Though all the experimental work to be described has been done on muscle extracts, or chopped muscle, there is every reason to believe that the fundamental chemical reactions are the same as those involved in the production of lactic acid in the living, intact muscle [Meyerhof, 1920, 1921 and Hill, Long and Lupton, 1923].

In the paper referred to above [Foster and Woodrow, 1924] some evidence was brought forward to show that the lactic acid inhibitor and antiglyoxalase are not identical and this is confirmed by the work to be described in the present paper.

Experimental details.

The method described by Dakin and Dudley [1913, 3; 1914] for the detection of glyoxalase and antiglyoxalase has been followed exactly, and, unless otherwise stated the procedure has been the same in all experiments. The glyoxalase solution used was obtained by treating minced chicken muscle with five times its weight of water and, after allowing the mixture to stand for an hour, filtering it through muslin. 50 cc. of the glyoxalase solution were allowed to act on 0·2 g. phenylglyoxal dissolved in 10 cc. water. The mixture was incubated over night at 37° with an excess of precipitated chalk and a few drops of toluene in a conical flask. The resulting mandelic acid was extracted with ether and its rotation measured, exactly in the way described by the above authors. When the muscle extract was allowed to act on the phenylglyoxal alone, a relatively large residue of white crystals of mandelic acid was left after the evaporation of the ether. This was dissolved in 15 cc. of water, boiled with a trace of charcoal and filtered. The rotation of the solution was measured in a 2 dm. polarimeter tube and the figures given in the tables are the rotations observed with mercury green light. The solutions examined were always perfectly clear and colourless. When a solution was tested for antiglyoxalase it was added to the glyoxalase some time, usually an hour, before the phenylglyoxal and the mixture kept at 37°. The control mixtures were always similarly incubated with an equal volume of saline.

Foster and Woodrow [1924] showed that a 70 % alcoholic extract of a dried pancreas preparation (the alcohol being subsequently removed by vacuum distillation, etc.) contained the lactic acid inhibitor, and it was an extract prepared in this way which was tested for antiglyoxalase. The same preparation of dried pancreas was used throughout, but the extract was made fresh for each experiment. In some cases (Exps. 2 and 3) it was found that the extract did have some antiglyoxalase action, but by exercising great care in preparing it, in particular by using alcohol accurately diluted to 70 % and centrifuging till the solution was perfectly clear, an extract was obtained which had no inhibiting action on glyoxalase (Exp. 4, Nos. 1 and 4).

Exp. 1. Chicken muscle used as source of glyoxalase. Two pancreatic extracts tested A and B. (B had been prepared some weeks earlier and kept at −7°.)

Table I.

No.	20 % extract of chicken muscle cc.	Calcium carbonate suspension cc.	Inhibitory factor	Phenyl-glyoxal g.	Toluene drops	Rotation	Notes
1	50	10	None	0·2	5	−3·16°	Control
3	50	10	5 cc. A	0·2	5	−3·46°	Duplicate
4	50	10	5 cc. A	0·2	5	−3·16°	No inhibition
5	50	10	5 cc. B	0·2	5	−3·3°	No inhibition
7	50	10	5 cc. A heated to 85° for 10 mins.	0·2	5	−3·01°	No inhibition
9	50	10	5 cc. A boiled	0·2	5	−3·27°	No inhibition

Incubated 1 hour

Incubated 20 hours

This solution therefore did not contain antiglyoxalase. Some of the same preparation was found to inhibit the formation of lactic acid in pigeon muscle by about 50 %[1].

Exp. 2. All details as in Expt. 1, except that the chicken muscle extract had been stored some time at −7° and had apparently lost some of its glyoxalase as the controls gave low rotations.

Table II.

No.	Inhibitor	Rotation	Notes
1	None	−0·98°	Control
2	None	−1·01°	Control
3	5 cc. 70 % alcoholic extract of pancreas	−0·81°	Slight inhibition
4	,, ,,	−0·90°	Slight inhibition

This preparation therefore did contain traces of antiglyoxalase.

Exp. 3. A 70 % alcoholic extract of pancreas was tested both for lactic acid inhibitor and for antiglyoxalase and chicken muscle was used in both cases.

A. *Lactic acid estimations.*

In each of four flasks were placed 10 cc. phosphate buffer solution p_H 7·5, 3 cc. saline or pancreatic extract and 1 cc. $N/10$ KCN and 4 g. minced muscle [see Foster and Woodrow, 1924]. Left at room temperature for 24 hours.

Table III.

No.	Inhibitor	% of lactic acid	Notes
1	3 cc. saline	(Lost)	Control
2	3 cc. saline	1·5	Control
3	3 cc. extract	0·911	40 % inhibition
4	3 cc. extract	0·787	47 % inhibition

There is therefore at least a 40 % inhibition of lactic acid production due to the extract.

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[1] Unpublished experiment by Mrs D. M. Needham.

B. *Glyoxalase estimations.*

Table IV.

No.	Inhibitory factor	Rotation	Notes
1	6 cc. saline	– 2·03°	Control
2	6 cc. saline	– 2·12°	Control
3	3 cc. extract + 3 cc. saline each	– 1·82°	16 % inhibition
4		– 1·64°	16 % inhibition
5	3 cc. insoluble residue + 3 cc. saline each	– 1·64°	26 % inhibition
6		– 1·42°	26 % inhibition
7	3 cc. extract + 3 cc. insoluble residue	– 1·55°	20 % inhibition
8	3 cc. extract + 3 cc. ether-soluble residue	– 1·45°	29 % inhibition

Not only was the final purified 70 % alcoholic extract of the pancreas tested for antiglyoxalase, but also the various fractions removed in course of preparation. The figures show that the extract contained some antiglyoxalase, but so did the residues from the ether and centrifuging. The inhibition of the glyoxalase by 70 % alcoholic extract is only 16 % of the total (as against 40 % of the lactic acid), and as the residues gave higher figures it seemed probable that more careful preparation of the final solution would remove all the antiglyoxalase.

A similar but more conclusive result was obtained in Expt. 4 in which a water extract was made of the residue of the gland insoluble in 70 % alcohol. The alcoholic extract was made in the usual way. The residue was then dried in a current of warm air to remove all traces of alcohol, and ground with sand and distilled water and left overnight. Then the mixture was centrifuged till a clear supernatant liquid was obtained. This was made up to 1 % with sodium carbonate and incubated at 37° to remove trypsin, as recommended by Dakin and Dudley [1913, 4]. It was found that there was some trypsin still left, but it has been shown that antiglyoxalase is distinct from trypsin (Dakin and Dudley [1913, 2] and Exp. 7 below). 5 cc. of this extract were equivalent to 1 g. of the dried gland.

Exp. 4.

Table V.

No.	Extract	Treatment	Rotation	Notes
12	5 cc. saline	—	– 3·21°	Control
11	5 cc. saline	—	– 3·12°	Control
1	5 cc. alcoholic extract	—	– 2·92°	Very slight inhibition
2		—	– 3·00°	
3	5 cc. alcoholic extract	Heated at 85° for 10 mins.	– 3·14°	No inhibition
4			– 3·04°	
5	5 cc. water ex-extract	—	– 0·52°	Almost complete inhibition
6		—	– 0·51°	
7	5 cc. water extract	Heated at 85° for 10 mins.	– 2·16°	Some destruction of antiglyoxalase
8			– 2·13°	
9	5 cc. water extract	Boiled for 10 mins.	– 1·72°	Some destruction of antiglyoxalase
10			– 1·64°	

These figures show that the extract made with 70 % alcohol was practically free from antiglyoxalase, whilst the watery extract was rich in this factor and could inhibit the glyoxalase. It would appear therefore that the lactic acid inhibitor is extracted by 70 % alcohol, whilst the antiglyoxalase is not.

SOME OBSERVATIONS ON ANTIGLYOXALASE.

In all the experiments with phenylglyoxal it was noticed that whenever there was any antiglyoxalase action, that is to say when no mandelic acid was produced after adding phenylglyoxal to a mixture of muscle extract and an extract of pancreas, there was a development of an orange coloration. The colour produced was always proportional to the degree of inhibition. If this was complete the colour produced during incubation of the mixture was a deep orange, and, with partial inhibition lighter colours were obtained. This phenomenon was noticed whatever pancreatic preparation was used, fresh fowl and ox pancreas, or a watery extract of the dried pancreas; and also when an alcoholic extract showed some inhibitory power, as in Exp. 2. The colour gradually increased during incubation.

The greater part of the colouring matter was precipitated during removal of the protein, by boiling after addition of ammonium sulphate, and the coagulum was always brightly coloured, but some colour persisted in the filtrate. Acidification with phosphoric acid produced no change but the ether solution and residue frequently turned red, and once or twice a blue colour was noticed at this stage. All traces of the colour disappeared on boiling with charcoal and the final solution was as clear and colourless as the controls. The control flasks in which mandelic acid was produced from the phenylglyoxal never showed any trace of this orange colour at any stage. Since this colour was invariably associated with a failure to convert the phenylglyoxal into mandelic acid it seemed worth while to investigate the connection between the two phenomena.

At first sight it seemed probable that the colour might be due to the excess of phenylglyoxal present when the glyoxalase was inhibited; but this can hardly be the case, as in the controls also there must be a large excess of unchanged phenylglyoxal present. A rotation of – 3° only means a conversion of 30 % of the 0·2 g. phenylglyoxal added to each flask into mandelic acid. The unchanged excess is precipitated during the boiling with ammonium sulphate [Dakin and Dudley, 1913, 1].

Then it was found that on incubating a pancreatic extract with phenylglyoxal, in the absence of any muscle extract or any other tissue preparation, the mixture turned the same deep orange colour and an orange precipitate was formed. Evidently there was some factor in pancreatic extract which could bring about this change in the phenylglyoxal. Is the apparent "antiglyoxalase" action of pancreatic preparations due, not to their inhibitory action on the glyoxalase, but to the fact that they contain something capable of changing phenylglyoxal into a highly coloured body which cannot give rise to mandelic acid? In other words, is their action not on the glyoxalase but on the phenylglyoxal? It is worth noting that in every experiment on antiglyoxalase reported in their original paper Dakin and Dudley [1913, 4] used phenylglyoxal as substrate and not methylglyoxal.

In the experiments described below some evidence is brought forward to show that pancreatic extracts do in some way change the phenylglyoxal. It cannot, of course, be said that they have no action on glyoxalase, and therefore that "antiglyoxalase" does not exist, until the action of pancreatic extracts has also been tried on methylglyoxal. It may be mentioned in passing, however, that it is difficult to imagine the significance and function of an antiglyoxalase since it is not found in the blood stream and the removal of the pancreas does not increase the glyoxalase action of the tissues [Dakin and Dudley, 1913, 4].

Experimental details.

If by previous incubation with pancreatic extract the phenylglyoxal has undergone some change, it will not give rise to mandelic acid when subsequently added to a glyoxalase preparation even if all the antiglyoxalase factor is removed. This was the principle underlying the following experiments. After incubation overnight the mixture of phenylglyoxal and a pancreatic extract, which was always orange, was boiled to destroy any antiglyoxalase and then added to a glyoxalase solution prepared from chicken muscle in the usual way. In order to demonstrate that any change in the phenylglyoxal was not due to the boiling, the solution (0·2 g. in 10 cc.) was boiled before being added to the control flasks. It was shown repeatedly that the phenylglyoxal was in no way affected by boiling or even autoclaving (Exp. 7). There were always controls with and without pancreatic extract as shown in the following table:

Exp. 5. Pancreatic extract 20 cc. = 1 g.

Table VI.

No.	Extract added	Treated	Rotation	Notes
1	20 cc. saline	None	−3·02°	Controls
2	20 cc. saline	None	−2·91°	Controls
3	20 cc. water extract	Raw	+0·01	Orange colour and inhibition
4	20 cc. water extract	Raw	+0·07°	
5	20 cc. water extract	Incubated with the phenylglyoxal 24 hours and then boiled 13 mins.	−0·23°	Orange colour and inhibition
6	20 cc. water extract		−0·25°	
7	20 cc. water extract	Boiled 13 mins.	−0·16°	Orange colour and inhibition
8	20 cc. water extract	Boiled 13 mins.	−0·39°	

There is therefore no mandelic acid formed from the phenylglyoxal, which was first treated with the pancreatic extract; but this is no evidence that the effect was due to destruction of the phenylglyoxal since the addition of boiled extract to the mixture containing glyoxalase also prevents the formation of mandelic acid. This means that either the phenylglyoxal was unchanged by the previous action of the pancreatic extract, which seems unlikely in view of the marked change in colour and the development of precipitate, or that the "anti" factor, whether it acts on the phenylglyoxal or glyoxalase, is thermostable. Dakin and Dudley [1913, 4] found their antiglyoxalase preparations destroyed by heating to 85° for 10 minutes and this was confirmed in the case of a very weak antiglyoxalase solution (see Exp. 4).

It is generally agreed that any factor of an enzyme character would be destroyed by autoclaving even if protected by other substances, such as protein, or by high concentration. Exp. 5 was therefore repeated and all the various solutions were autoclaved at 120° for 20 minutes. It was necessary to show, first of all, that the phenylglyoxal itself was unaffected by autoclaving, and this was done by comparing the effect of glyoxalase on autoclaved and unautoclaved phenylglyoxal. A mixture of rat muscle and liver was used as the source of glyoxalase.

Table VII.

No.	20 % extract of rat liver and muscle	Phenyl-glyoxal 2 %	Treatment	Rotations
1	50 cc.	10 cc.	Control	−2·67°
2	50 cc.	10 cc.	Autoclaved	−2·95°

The phenylglyoxal solution can therefore be autoclaved with impunity.

Exp. 7. Chicken muscle as source of glyoxalase.

Table VIII.

No.	Extract added	Treatment	Rotation	Notes
1	10 cc. saline	None	−2·42°	Control
2	10 cc. saline	None	−2·40°	Control
3	10 cc. water extract	Incubated 24 hrs. with 0·2 g. phenylglyoxal and then the whole autoclaved	−0·09°	Inhibition. The mixture was bright orange before addition of muscle extract
4			−0·09°	
5	10 cc. water extract	None	−0·10°	Inhibition. Orange colour developed during incubation
6		None	−0·05°	
7	10 cc. water extract	Autoclaved before use	−0·92°	Some development of orange colour and partial inhibition
8		Autoclaved before use	−0·81°	

It is obvious that even autoclaving has not completely destroyed the inhibitory factor whatever it is. It is extremely improbable that anything of an "organised" nature would have survived such treatment. All trace of trypsin has gone, though it is difficult to destroy by boiling alone in such high concentration. It must be some thermostable substance present in the pancreatic extract which is producing the effect.

In order to make sure that the results observed were not due to differences of reaction, though this was very improbable in view of the excess of chalk used in every flask, the experiment was repeated exactly, except that every solution was neutralised to p_H 7·0 both before and after autoclaving. The result was the same as shown by Table VIII.

Exp. 8.

Table IX.

No.	Extract	Treatment	Rotation	Notes
1	10 cc. saline	—	−2·39°	Control
2	10 cc. water extract	Incubated overnight with phenylglyoxal and then autoclaved	−0·16	Inhibition
3	10 cc. water extract	Raw	−0·13°	Inhibition
4	10 cc. water extract	Autoclaved	−0·80°	Partial inhibition

Owing to the difficulty of removing the factor after its action on phenylglyoxal, these experiments do not definitely prove that its action is on the phenylglyoxal rather than the glyoxalase; but there are two facts which make this extremely probable. One is the development of the orange colour, which invariably accompanies inhibition, by the action of the pancreatic extract on the phenylglyoxal alone. The other is that the inhibition produced by the autoclaved extract on the whole mixture is only partial (as shown by Exp. 4, Nos. 7 and 8), while the inhibition produced by its action on the phenylglyoxal is complete.

Is METHYLGLYOXAL THE PRECURSOR OF LACTIC ACID IN MUSCLE?

In the paper already referred to [Foster and Woodrow, 1924] the suggestion is made that certain facts of muscular metabolism, in particular the liberation of lactic acid at special surfaces where it is required for a special purpose in the series of phenomena that result in muscular movement, would be more easily explained if there were any evidence to show that the muscle has a specialised carbohydrate metabolism of its own; that is to say, if the sequence of chemical changes, which take place in the production of lactic acid from the carbohydrate stores of the muscle, were not necessarily the same as those taking place in other organs, where the lactic acid is merely a stage on the way to the complete combustion of glucose to carbon dioxide and water, with the liberation of all its available energy. In the muscle lactic acid is needed in relatively large quantities at a given surface at a given moment, and is produced as the result of a special stimulus, normally through a nerve. If there were this alternative course in the breakdown of glucose the mechanism controlling or inhibiting these changes would be different from that in an organ not so specialised; the liver for example. As already tentatively suggested such a differentiation would help to explain some of the anomalies of the diabetic condition.

It must be admitted that up to the present there is very little evidence in favour of this hypothesis, but one significant fact has accidentally come to light in the course of the work described in the earlier part of this paper. Some months ago the author (in collaboration with Mr C. E. Woodrow) made repeated attempts to test the pancreatic extract for the presence of antiglyoxalase in the way described above. Time after time the same difficulty occurred, and the experiments were useless because the controls were always negative. When a muscle extract prepared in the routine way was added to phenylglyoxal no mandelic acid was obtained. This was repeated five or six times on different specimens of phenylglyoxal and no mandelic acid was ever produced. Later when another muscle extract (chicken) was used as the source of glyoxalase no further difficulty was experienced, and it was always possible to get an extract able to convert phenylglyoxal into mandelic acid. The reason for our lack of success in all the earlier experiments was that we had always used *rabbit* muscle. It is now clear, as the experiments described below prove, that rabbit *muscle* contains no glyoxalase.

It seemed impossible that the original discoverers of glyoxalase should not have found this exception to the general rule that, with the exception of the pancreas and certain specialised glands, all tissues contain glyoxalase, including the muscle of various animals, *e.g.* dog, fowl, cat, etc. An examination of their results [Dakin and Dudley, 1913, 1, 2, 3 and 4] shows that whenever the rabbit was the animal selected they used a mixture of muscle and *liver*. As the experiments described in detail below show, rabbit liver is very rich in glyoxalase, while from the muscle it is completely absent. This is a very significant fact.

Experimental results.

These experiments were all carried out in the routine way already described. In each case a 20 % extract of the muscle was used and the rotation is that obtained by the action of 50 cc. muscle extract on 0·2 g. phenylglyoxal. The final solution was always made up to 15 cc. and the reading taken in a 2 dm. polarimeter tube, mercury green being the source of light.

Table X.

Exp. no.	Source of glyoxalase	Rotation of mandelic acid	Notes
10	Rabbit muscle	−0·21°	No crystalline residue from the ether
	Rabbit muscle	−0·19°	
14	Rabbit liver	−2·50°	Typical crystals of mandelic acid
	Rabbit liver	−2·28°	
	Muscle from same animal	+0·44°	No crystalline residue
	Muscle from same animal	+0·39°	No crystalline residue
17	Rabbit liver	−2·17°	Mandelic acid crystals
	Rabbit liver	−2·26°	Mandelic acid crystals
	Muscle from same animal	+0·05°	No residue except traces of ammonium sulphate
	Muscle from same animal	+0·07°	

It is quite certain that rabbit muscle produces lactic acid in a perfectly normal way, in fact it is the muscle on which a great deal of experimental work has been done [Fletcher, 1911, 1913; Winfield and Hopkins, 1915]. The present author has frequently found that chopped rabbit muscle produces lactic acid in relatively large amount. The following figures will serve as examples. In each case the muscle was removed immediately after the death of the rabbit and rapidly minced. One fraction was at once ground in ice-cold alcohol and served as control, while the rest was left at room temperature for 24 hours in various buffered solutions and the lactic acid then estimated in the routine way [Foster and Woodrow, 1924]. These figures are selected at random from a large number of experiments carried out for another purpose.

Table XI.

% of lactic acid in control	% of lactic acid after incubation	Notes
0·164 (1) 0·164 (2)	0·523 (1) 0·664 (2)	Incubated in a "Ringer" solution containing sodium bicarbonate (1) or borate (2)
0·16 0·13	0·44 0·47	Incubated in a "Ringer" solution containing bicarbonate
0·18 0·19	0·46 0·46	Incubated in a "Ringer" solution containing bicarbonate
0·12 0·14	0·54 0·53	Incubated in a "Ringer" solution containing bicarbonate
0·16	0·673	Incubated in a "Ringer" solution containing bicarbonate

It follows from these experiments that in rabbit muscle lactic acid is produced without the action of glyoxalase and, presumably therefore, it does not arise from methylglyoxal. On the other hand, the liver of the same animal is rich in glyoxalase and there is every reason to suppose, as Dakin and Dudley suggested [1913, 1914], that it plays an important part in the conversion of ketonic aldehydes into the corresponding α-hydroxy-acids, and that lactic acid is produced in this way in this organ from methylglyoxal. This is at present an isolated instance and the muscle of certain other animals, mammals as well as birds, has definitely been shown to contain glyoxalase, but it is possible that there are other animals like the rabbit which have no glyoxalase in their muscles. However, even one exception is important since it shows that methylglyoxal is not necessarily a precursor of lactic acid. It is significant that the amount of mandelic acid obtained by Dakin and Dudley [1913, 4] when muscle was used as the source of glyoxalase, was a good deal lower than that obtained from the same weight of liver. For example, for the dog the rotations observed were:

Liver	– 2·3° to – 2·58°
Muscle	– 0·45° to – 1·13°
Heart and tongue	– 0·45°

It is possible that where glyoxalase is found in muscle it serves to catalyse the breakdown of glucose into carbon dioxide and water, through methylglyoxal, in the general metabolism of the muscle, but plays no part in the special production of lactic acid as the result of a nerve stimulus, which results in contraction.

SUMMARY.

1. The factor found in the pancreas and able to inhibit the production of lactic acid by chopped muscle *in vitro* is not antiglyoxalase.

2. Some evidence is brought forward to show that the "antiglyoxalase" action is probably due to the action of some factor in the pancreas on phenylglyoxal rather than on the glyoxalase.

3. It is shown that rabbit muscle does not contain glyoxalase and the significance of this in the carbohydrate metabolism of muscle is discussed.

I wish to thank Dr Dudley for his interest and for the hospitality of his laboratory at one stage of this work, and Sir F. G. Hopkins for his continuous help and encouragement.

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CXVIII. A METHOD FOR THE ESTIMATION OF IODINE IN THYROID GLAND.

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(*Received August 6th, 1925.*)

An examination of the published methods for the estimation of iodine in tissues has shown certain objections to their use for the special purpose of determining the iodine in thyroid gland.

Iodine is very reactive and is volatile so that its estimation is not easy. Many methods for its estimation have been put forward, the most important of which are briefly criticised below.

In comparing them however with the method to be given later, attention is drawn to the desirability of avoiding all processes involving unknown factors, and those subject to a large personal equation such as the colorimetric.

Baumann's method and its large number of modifications consist in the fusion of the gland with sodium hydroxide and potassium nitrate, followed by acidification and the extraction and estimation of the liberated iodine. The objection to all modifications of this method is that the effervescence which occurs on acidification causes an indefinite loss of iodine.

Hunter's method destroys the organic compounds by heating with a mixture of potassium and sodium carbonates and nitrates; the dissolved melt is treated while still alkaline with sodium hypochlorite; the solution is acidified and boiled to expel excess of chlorine, potassium iodide added and the iodine titrated with thiosulphate. The objections are the possible formation of oxychlorine compounds (Kendall) which would invalidate the result; and also the fact that the fusion process is inadequate with organic iodine compounds such as are met with in thyroid. Kelly and Husband also find that the process gives low results with seaweed.

Kelly and Husband's [1924] modification of Kendall's process was published while the present work was in progress. It consists of alkali fusion with sodium hydroxide and completion of the oxidation with potassium nitrate as in the Baumann method; acidification with phosphoric acid, and prolonged boiling to get rid of the nitrous acid. Sulphite is added and the SO_2 boiled off and then bromine is added, excess of which is also got rid of by prolonged boiling. Potassium iodide is then added and the liberated iodine is titrated.

One objection, as in the Baumann method, is the possible loss of iodine in the neutralisation of the mixture with acid, which liberates nitrous acid and much carbon dioxide.

Another objection is the fact, easily demonstrable in a test-tube, that bromides liberate iodine from potassium iodate in the presence of acid; an indefinite quantity of bromide is necessarily formed in the process of oxidation by the bromine.

The prolonged boiling and bromine vapour also present certain practical objections.

In Fellenberg's process [1924] the fusion process differs from the foregoing in that no potassium nitrate is added; but iodine is estimated colorimetrically. It is not intended by this author to be exactly quantitative since the results are multiplied by $\frac{5}{4}$ to allow for loss. It is a good method for the detection of minute amounts of iodine.

The method put forward depends upon the destruction of organic matter by fusion with alkali and oxidation of the resulting iodide to iodate with permanganate solution; excess of the latter is removed with animal charcoal, and after adding iodide the liberated iodine is titrated with thiosulphate.

The complete process is as follows:

0·25 g. of finely powdered dry thryoid (1) is weighed and introduced into a nickel crucible of 150 cc. capacity; 10 cc. 50 % sodium hydroxide (2) is added and mixed by rotating the crucible; it is covered with a lid having a hole $\frac{1}{8}''$ in the centre and heated slowly till the water is evaporated; it is next heated in an oven considerably below red heat but hot enough to give a clear fusion product in about an hour (3); the crucible is next heated over a large free flame protected so that the crucible and the lid can be rapidly raised to a dull red heat, which is maintained $\frac{1}{2}$—1 minute (4).

Allow the crucible to cool, add about 70 cc. water and place on a warm plate for about an hour, when gentle stirring will bring the whole into solution (5); transfer to a 250 cc. Erlenmeyer flask, add three drops 10 % sodium sulphite solution (6); acidify with 50 % sulphuric acid (7) from a burette, about 13 cc. being required (test acidity with a narrow strip of litmus paper mounted on a holder); add 3 cc. in excess, cool and add 5 cc. approx. $N/10$ permanganate; after 3 minutes add a suspension of charcoal (8); when decolorisation is complete filter through a suction filter (9) washing with a little water, or through a pleated filter paper (10); add a crystal of potassium iodide and titrate immediately with freshly prepared $N/100$ thiosulphate, adding starch only towards the end of the titration.

Each 1 cc. thiosulphate × 85 gives the milligrams of iodine in 100 g. of dried gland when 0·25 g. is taken for the analysis. It is found convenient to work with batches of six thyroids.

NOTES ON THE METHOD.

(1) The thyroid should pass a 60 sieve.

(2) The soda should be free from nitrate and from chloride, the commercial "Soda by alcohol" answers well.

(3) Potassium nitrate must not be added.

(4) Prolonged heating at red heat causes a slight loss.

(5) A few carbon particles remain which do not affect the result. See Exp. 7 below in which a small amount of charcoal is added at the initial stage.

(6) This does not apparently affect the result but is added as a precaution against the possibility of iodine being liberated at this stage by traces of impurity such as iron.

(7) The sulphuric acid must be chemically pure.

(8) Shake 20 g. animal charcoal with 1000 cc. water and 2 cc. sulphuric acid. The British Drug Houses "Animal charcoal purified by acid" is satisfactory. Use a pipette with a very wide jet for measuring rapidly before sedimentation takes place. The amount required is determined by that which will reduce 5 cc. acidified $N/10$ permanganate in not less than 5 or more than 20 minutes: this is usually 6 cc.

(9) The filtrate must be inspected very carefully to see that it is absolutely free from colour and from minute particles of carbon.

(10) If a pleated filter is used it is found more convenient, instead of washing, to weigh against another paper as a counterpoise and make the small correction for the amount of fluid on the paper and in the flask, *e.g.* if the weight on the paper is 3 g. and that in the flask 0·5 g. the corrected figure will be 113·5/110 with an amount of fluid of 110 cc.

Suitable suction filters are the Jena Sintered Glass 17G 4/< 7 filters.

The permanganate may be added to the alkaline solution instead of after acidification if desired, in which case addition of sulphite is unnecessary.

Iron should be removed from the solution if the thyroids contain a large excess of blood by adding to the solution after acidification enough soda to make just alkaline and after standing some hours filtering off the precipitated iron, acidifying, adding 3 cc. of 50 % acid and proceeding as above.

Test experiments with experimental mixtures.

Solutions used. Potassium iodide $N/200$. Thiosulphate $N/100$ made fresh each day by diluting 10 cc. of $N/10$ to 100 cc. The $N/10$ thiosulphate was preserved with a crystal of thymol and 25 cc. were equivalent to 0·1226 g. $K_2Cr_2O_7$.

In tests 1–11, 1 cc. of $N/200$ KI was measured by means of a 1 cc. D and R pipette the standardisation of which was checked by weighing. The theoretical titration figure in these experiments (1–11) was therefore 3·00 cc.

Exps. 1–9 consisted of the latter half of the process; *i.e.* without addition of alkali and the fusion process. Variations in amount of reagents and other factors were introduced into the test processes below to show the flexibility of the process in allowing such variation:

(1) *The amount of acid* (instead of 3 cc.) was varied as follows:

	Acid (cc.)	Titre
	0·5	2·60
	1·0	2·86
	2·0	3·02
	4·0	2·99
	5·0	3·03
Blank	12·0	0·00

The amount of acid must therefore be at least 2 cc.

(2) *The amount of permanganate* (instead of 5 cc.) was varied as follows:

$KMnO_4$ (cc.)	Titre
0·5	2·93
1·0	3·02
2·0	2·97
5·0	3·01
10·0	2·97

The amount may vary considerably.

(3) *Temperature of action of* $KMnO_4$:

	Titre
Ordinary	3·01
Hot 60—70°	2·95
Hot 60—70°	2·97

The temperature is not important.

(4) *Time of action of* $KMnO_4$:

	Titre
Immediate	2·99
1 min.	3·02
2 mins.	3·04
5 ,,	3·07
90 ,,	3·07
90 ,,	3·06

The permanganate oxidises the iodide immediately.

(5) *The amount of charcoal* (instead of 6 cc.) was varied as follows:

cc.	Titre
5	3·05
7	3·02
9	3·00

The amount of charcoal may vary within reasonable limits.

(6) *The amount of iodine*:

cc.	Titre
1	3·01
2	5·97

Also see No. 10.

(7) *Charcoal* (0·5 cc. suspension) *present at start*:

Titre 3·03

A small quantity of carbon present in the process will not affect the result.

(8) *Time in contact with the charcoal after the decoloration is completed*:

mins.	Titre
30	2·97
120	2·98

The charcoal does not reduce the iodate.

(9) *Effect of salts*:

	Titre
10 cc. NaOH neutralised, 2·5 cc. excess acid	3·01
10 cc. NaOH neutralised, 2·5 cc. excess acid (blank)	0·00
10 cc. NaOH neutralised after previously adding 5 drops of 10 % sulphite...	3·02

The salts present in the actual experiment do not affect the result.

(10) The following were experimental mixtures of the iodide solution and 0·25 g. of meat mixed and treated by the complete process as above:

cc.	Titre
1	2·96
2	5·93

The amount of iodine may vary widely.

(11) *Temperature and amount of heating varied*:

30 mins. slow heating till clear, then as follows:	Titre
1. Melt, not red heat	2·90
2. Red hot 1 min., quick heating	3·00
3. Red hot 3 mins.	2·98
4. Red hot 10 mins.	2·91
9. Blank with starch	0·01
10. Blank with meat	0·04

The amount of heating is important. Also see Exp. 12.

(12) *Thyroid gland*:

	Titre
Just red hot	1·65
Red hot 1 min.	1·62
Red hot 5 mins.	1·62
Red hot 10 mins.	1·61
Red hot 30 mins.	1·61
Well heated but always under red heat	1·64

There is a slight loss by prolonged heating at red heat.

The above show that there is considerable liberty allowed in the amount of reagents and other factors of the method.

Duplicate experiments give results which are correct to one drop of titrating solution. All duplicate results were obtained at different times.

Over 100 human thyroid glands have been analysed by the method and results obtained ranging from 5–520 mg. per 100 g. of dried tissue; and 0·4–52·5 mg. per whole gland obtained, details of which are being published in the *Journal of Mental Science*.

The above work was carried out in the laboratories of the Joint Board of Research for Mental Diseases, City and University of Birmingham.

My best thanks are due to Sir Frederick Mott, K.B.E., M.D., F.R.S., for suggesting this work.

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CXIX. THE SEXUAL GLANDS AND METABOLISM.

IV. THE INFLUENCE OF INJECTIONS OF EMULSIONS OF TESTES AND PROSTATE AND OF INSULIN-LIKE TESTICULAR EXTRACTS UPON THE NITROGEN METABOLISM OF NORMAL, CASTRATED AND THYROIDECTOMISED RABBITS.

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(*Received August 12th, 1925.*)

I. Historical.

Castration produces a fall in the nitrogen metabolism of most dogs and rabbits [Korenchevsky, 1925, 1]. Dog's testes contain specific hormones which probably prevail when injected in the form of testicular emulsion [Korenchevsky, 1925, 2]. This is indicated by an increase in the nitrogen metabolism of most dogs after these injections. Thyroidectomy in dogs does not change this effect. It seems that in dogs the sexual hormones do not produce their influence upon nitrogen metabolism by means of the thyroid or at any rate not exclusively. On the other hand, Korenchevsky and Carr [1925] found that homologous testis emulsion when injected subcutaneously into rabbits slightly decreased the nitrogen metabolism during the period of injections and in most cases during the succeeding 5–9 days. This effect of testis emulsion cannot, however, be considered to be a specific one because of the similar effect of kidney emulsion.

An emulsion of prostate, injected alone or in combination with testis, increased the nitrogen metabolism up to 11 %. The rise in N output was manifested more particularly during the 2 or 3 days following the period of injections.

The diminished N metabolism after the injection of testis emulsion in the rabbit was contrary to expectation because castration also produces a fall in the nitrogen metabolism [Korenchevsky, 1925, 1]. Therefore we were inclined to attribute these contradictory results chiefly to the possible presence in the rabbit's testis of two different principles, one increasing, the other decreasing nitrogen metabolism, the latter prevailing when an emulsion of this organ is injected. Ashby [1923], Baker, Dickens and Dodds [1924], Ivy and Fisher [1924] and Best, Smith and Scott [1924] have found that various tissues, including kidney, contain an insulin-like substance. And according to Fitz, Murphy and Grant [1922], Wilder, Boothby, Barborka, Kitchen and Adams [1922], MacLeod and Allan [1923] and Kudrjavzeva [1925] injections of insulin lower the nitrogen metabolism. Therefore, if testes contain an insulin-like factor, this latter substance may partly be the cause of the decreased nitrogen metabolism.

The experiments to which we shall refer in this paper seem to justify this theory.

II. The present investigation.

Technique.

Twenty-three experiments were performed on 12 rabbits, of which 4 were castrated (Nos. 14, 22, 26, 33), 4 thyroidectomised (Nos. 24, 31, 38, 39), 3 both thyroidectomised and castrated (Nos. 23, 17, 40) and 1 normal (No. 41). The technique of the metabolism experiments, the preparation of the emulsions of prostate and testis and the amounts injected are the same as those described in a previous paper [Korenchevsky and Carr, 1925].

Mr F. H. Carr at the laboratories of the British Drug Houses isolated insulin-like substances from bull's testes and kindly supplied us with the amount necessary for our experiments—we wish to thank him and his firm for this as well as for the following data concerning the method of purification and the influence of these substances upon the blood-sugar of rabbits, the latter being determined by Dr S. W. Underhill.

The method of isolation briefly consisted in the precipitation of alcoholic extracts of bull's testes at different p_H values, namely, p_H 6·8 (testicular extract A), p_H 5·0 (testicular extract B) and p_H 4·0 (testicular extract C). These three substances were converted to hydrochlorides by Dudley's method. They reduced the blood-sugar content of rabbits as follows:

Testicular extract A.	2·5	mg. per kg.	reduced	the blood-sugar	to	55	mg.	per 100 cc.
	4·0	,,	,,	,,	,,	74	,,	,,
Testicular extract B.	0·15	,,	,,	,,	,,	44	,,	,,
	0·25	,,	,,	,,	,,	50	,,	,,
	0·4	,,	,,	,,	,,	51	,,	,,
Testicular extract C.	2·0	,,	,,	,,	,,	45	,,	,,
	3·0	,,	,,	,,	,,	54	,,	,,

For our experiments these three fractions were each dissolved in sterile distilled water and injected subcutaneously in amounts varying between 0·8 mg.–2·3 mg. per diem and per kg. of the rabbit's weight. The amounts injected per diem and kg. are given in the tables. Each experiment was divided into four periods, namely, the first control period of 3 days before the injections, the period of injections lasting 3 days, followed by two or three control periods, each of which usually lasted 3 or sometimes one of them only 2 days. When the doses were increased and we feared convulsions might follow, the injection period only lasted for 1 day. For this same reason in

Exps. 2, 7, 12, 15 and 16 the rabbits were injected with small doses (about 0·8 mg. per kg.) for 3 days. Five days after the injection period, when the nitrogen metabolism of the thyroidectomised animals was still below normal, another larger injection was given once only.

In normal or castrated animals fractions A, B and C were injected in the same or larger amounts.

In order not to increase the amount of tabular matter, firstly, we do not include the experiments on testicular extract A, because its activity was small, nor the experiments on the injections of a mixture of A, B and C extracts, because the results obtained were nearly identical with those with B extract. Secondly, though the nitrogen intake in the food and output in the urine and faeces were estimated carefully as in our previous work, they are not included in the tables. The daily intake of nitrogen was always the same during a whole experiment. It only varied slightly in different experiments, because the cabbage could be stored for the length of one experiment only (about 18 days), but not for all the experiments on the same animal [for further details *see* Korenchevsky and Carr, 1925, pp. 77, 78; Korenchevsky, 1925, 3].

Description of the Experiment.

The actual figures and details of the experiments are given in Tables I and II and the results obtained are summarised in Tables III and IV. In the latter the figures show the average percentage change above (+) or below (−) normal in the urinary nitrogen excretion and in the urinary flow during the period of injections and the two or three following control periods, compared with that of the same animal during the first control period before the injections. In Tables III and IV the average percentage changes in thyroidectomised animals are compared with those in normal or castrated animals with thyroids. The average figures of the urinary nitrogen and flow of the latter animals were taken from experiments published previously [Korenchevsky and Carr, 1925]. As was mentioned above, the experimental technique was exactly the same in both.

Injections of Testis Emulsion.

The details of these experiments on rabbits 23 and 24 are given in Table I (Exps. 1 and 3) and Table II (Exp. 10). Before the period of injections in Exp. 1 the nitrogen balance was positive, but was negative in this period in Exps. 3 and 10. The results of all three experiments were almost identical, namely, a quite definite decrease in the nitrogen excreted in the urine (15·5, 14·5 and 7·2 % respectively below normal) and an increase in the nitrogen balance. This was always most pronounced, and in two experiments was only present in the control period following the injections.

A comparison of the results obtained on animals with thyroids and on those without thyroids (see Table III) shows that the injection of testis

Table I. *The influence of subcutaneous injections of insulin-like extracts from testis a of emulsions of testis and of prostate upon the nitrogen metabolism of rabbits.*

No. of experiment and rabbit	No. of period	Weight of rabbit g.	Volume of urine excreted cc.	Nitrogen in urine g.	Balance of nitrogen g.	Remarks
Exp. 1						
Rabbit 23 (thyroidectomised and castrated)	I	3575	448	9·43	+0·11	I control period
	II	3577	462	9·57	−0·13	Injection of testis emulsion
	III	3605	375	8·27	+1·17	II control period
	IV	3640	348	8·81	+0·63	III control period
	V	3655	504	8·69	+0·78	IV control period
Exp. 2						
Rabbit 23	VI	3490	651	8·07	−1·56	I control period
	VII	3485	628	7·56	−1·05	Injection of 0·9 mg. testis extrac
	VIII	3480	571	7·60	−1·09	II control period
	IX	3480	562	7·48	−0·97	III control period
	X	3500	518	7·17	−0·66	Injection of 1·7 mg. testis extrac
	XI	3475	777	7·83	−1·32	IV control period
Exp. 3						
Rabbit 23	XII	3420	611	7·58	−1·11	I control period
	XIII	3403	565	7·85	−1·38	Injection of testis emulsion
	XIV	3420	420	6·48	−0·01	II control period
	XV	3420	502	6·93	−0·46	III control period
Exp. 4						
Rabbit 38 (thyroidectomised)	I	2695	418	6·50	−0·52	I control period
	II	2695	385	6·72	−0·74	Injection of prostate and tes emulsion
	III	2685	371	6·51	−0·53	II control period
	IV	2680	373	6·72	−0·74	IV control period
Exp. 5						
Rabbit 40 (thyroidectomised and castrated)	I	2560	424	6·25	−0·46	I control period
	II	2565	434	6·43	−0·64	Injection of prostate emulsion
	III	2545	485	6·24	−0·45	II control period
	IV	2545	428	6·14	−0·35	III control period
Exp. 6						
Rabbit 17 (thyroidectomised and castrated)	I	2590	488	8·71	−0·54	I control period
	II	2627	286	7·98	+0·19	Injection of emulsion of prost and testis
	III	2620	518	8·23	−0·06	II control period
	IV	2620	441	8·57	−0·40	III control period
	V	2595	434	8·56	−0·39	IV control period
Exp. 7						
Rabbit 17	VI	2580	390	7·36	−0·14	I control period
	VII	2550	462	7·34	−0·12	Injection of 0·8 mg. of testis tract B
	VIII	2565	329	6·99	+0·23	II control period
	IX	2560	392	6·93	+0·29	III control period
	X	2520	616	6·92	+0·30	Injection of 1 mg. of testis extract
	XI	2545	273	6·16	+1·06	IV control period
Exp. 8						
Rabbit 17	XII	2550	401	7·90	−0·15	I control period
	XIII	2580	380	7·80	−0·05	Injection of emulsion of prosta and testis
	XIV	2585	343	7·57	+0·18	II control period
	XV	2565	406	7·70	+0·05	III control period

TE. In this and the following tables figures showing N intake and N content of faeces are omitted for brevit sake. The figures are calculated for 1 week so as to be comparable. The figures for insulin-like extrac represent the amount injected per diem and kg.

Table II. *The influence of subcutaneous injections of insulin-like extracts from testis and of emulsions of prostate and of testis upon the nitrogen metabolism of rabbits.*

No. of experiment and of rabbit	No. of period	Weight of rabbit g.	Volume of urine excreted cc.	Nitrogen in urine g.	Balance of nitrogen g.	Remarks
Exp. 9						
Rabbit 24 (thyroidectomised)	I	3185	530	8·49	+0·62	I control period
	II	3215	560	8·37	+0·74	Injection of prostate emulsion
	III	3168	601	8·52	+0·59	II control period
	IV	3195	478	8·71	+0·40	III control period
	V	3275	411	8·36	+0·75	IV control period
Exp. 10						
Rabbit 24	XII	3495	527	9·32	−0·92	I control period
	XIII	3485	548	8·92	−0·52	Injection of testis emulsion
	XIV	3495	462	8·65	−0·25	II control period
	XV	3485	586	8·82	−0·42	III control period
Exp. 11						
Rabbit 31 (thyroidectomised)	I	2555	537	7·65	−0·29	I control period
	II *b*	2567	392	5·70	+1·66	Injection of 1·4 mg. testis extract B
	V	2565	553	7·51	−0·15	IV control period
Exp. 12						
Rabbit 31	VI	2542	504	7·25	−1·93	I control period
	VII	2505	537	7·25	−1·93	Injection of 0·8 mg. testis extract B
	VIII	2520	392	6·36	−1·04	II control period
	IX	2545	436	6·28	−0·96	III control period
	X	2550	392	5·73	−0·41	Injection of 1·0 mg. testis extract B
Exp. 13						
Rabbit 31	XII	2408	488	7·33	−1·41	I control period
	XIII	2370	567	7·34	−1·42	Injection of prostate emulsion
	XIV	2330	553	7·25	−1·33	II control period
	XV	2320	495	7·15	−1·23	III control period
Exp. 14						
Rabbit 33 (castrated)	I	2570	586	8·72	−0·18	I control period
	II	2555	525	8·48	+0·06	Injection of 1·4 mg. testis extract C
	III	2560	478	9·07	−0·53	II control period
	IV	2580	455	8·76	−0·22	III control period
	V	2565	436	8·41	+0·13	IV control period
Exp. 15						
Rabbit 33	VI	2627	495	8·28	−0·52	I control period
	VII	2620	490	7·80	−0·04	Injection of 1·2 mg. testis extract C
	VIII	2640	403	7·91	−0·15	II control period
	IX	2640	488	8·00	−0·24	III control period
	X	2650	392	8·06	−0·30	Injection of 2·3 mg. testis extract C
	XI	2645	455	7·80	−0·04	IV control period
Exp. 16						
Rabbit 14 (castrated)	I	3285	455	8·67	+0·39	I control period
	II	3295	420	8·40	+0·66	Injection of 0·9 mg. testis extract B
	III	3310	378	8·52	+0·54	II control period
	IV	3310	413	8·30	+0·76	III control period
	V	3300	546	8·30	+0·76	Injection of 1·3 mg. testis extract B
	VI	3305	378	8·19	+0·87	IV control period
Exp. 17						
Rabbit 26 (castrated)	I	2197	432	7·10	0	I control period
	II	2135	487	7·11	−0·01	Injection of 1·3 mg. testis extract B
	III	2160	348	7·66	−0·56	II control period
	IV	2160	387	7·10	0	III control period
	V	2165	395	6·98	+0·12	IV control period
Exp. 18						
Rabbit 41 (normal)	I	2880	443	9·04	−0·23	I control period
	II	2895	364	8·29	+0·52	Injection of 1·5 mg. testis extract B
	III	2890	280	9·03	−0·22	II control period

Table III. *Average percentage change from the normal in the N output in the urine of rabbits after injection of insulin-like extracts from testis and of different emulsions.*

Injection	Number of experiments averaged	Thyroid present (+) or removed (−)	Experimental periods			
			Injection period 3 days	II control period 2 days	III control period 3 days	IV control period 2 days
Kidney emulsion	3	+	− 4·4	− 4·9	−4·8	.
Testis emulsion	4	+	− 4·6	− 2·5	−2·4	.
	3	−	+ 0·2	−12·4	−7·1	−7·9
Prostate emulsion	4	+	+ 3·1	+ 9·0	+1·5	+3·3
	3	−	+ 0·5	− 0·2	−0·5	−1·6
Prostate and testis emulsion	5	+	+ 0·5	+ 9·2	+3·3	+1·3
	3	−	− 2·1	− 3·3	−0·3	−1·7
Testis insulin B per diem and kg.						
0·9 mg.	1	+	− 3·2	− 1·8	−4·3	.
0·8	2	−	0	− 8·6	−0·7	.
1·3–1·5	3	+	− 4·2	− 0·8	.	.
1–1·4	3	−	−17·5	−26·9	.	.
Testis insulin C per diem and kg.						
1·2–2·3 mg.	3	+	− 3·9	− 2·0	−1·7	.
0·9–1·7	2	−	− 8·8	− 4·7	−7·4	.

Table IV. *Average percentage change from the normal in urinary flow of rabbits after injection of insulin-like extracts from testis and of different emulsions.*

Injection	Thyroid present (+) or removed (−)	Injection period	II control period	III control period
Kidney emulsion	+	− 5·5	− 4·6	+ 5·0
Testis emulsion	+	− 8·8	−15·8	−12·0
	−	− 0·2	−19·9	− 9·6
Prostate emulsion	+	− 5·1	− 0·1	− 8·6
	−	+ 8·1	+13·7	− 2·4
Prostate and testis emulsion	+	− 2·4	−12·7	−13·3
	−	−18·2	− 6·6	− 6·4
Testis insulin B	+	+ 1·8	−22·5	− 9·8
	−	+ 1·5	−17·9	− 6·8
Testis insulin C	+	−10·4	−15·0	−12·4
	−	−12·0	−15·9	−13·7

emulsion decreased the nitrogen metabolism of thyroidectomised rabbits much more than that of normal or castrated animals.

Urinary flow was also decreased in the period following the injections, but, as the corresponding figures in Table IV show, there was no essential difference in this respect between the animals with and those without thyroids. This decrease in flow is incapable of interpretation, because we have not measured the water expired nor the water content of the faeces. Moreover, only in one experiment (No. 1) of the three was there an increase in weight. Therefore, there are no definite indications that the water is retained in the organism. The percentage of nitrogen excreted in the dried faeces was slightly increased in two experiments (Nos. 3 and 10) and slightly decreased in one (No. 1).

Injections of Prostate Emulsion.

The details of these experiments on rabbits Nos. 24, 31 and 40 are given in Table I (Exp. 5) and Table II (Exps. 9 and 13). These figures and a comparison of the results obtained on rabbits with thyroids with those on rabbits without thyroids show that the increase in nitrogen metabolism produced by the injection of prostate emulsion in animals with thyroids was not observed in the thyroidectomised animals.

Table IV shows that the excretion of urine during the injections of prostate emulsion and the succeeding period was increased in all thyroidectomised animals, whereas that of rabbits with thyroids was on an average slightly decreased.

There were no constant changes in the percentage of nitrogen excreted in the faeces as a result of the prostate injections.

Injections of a Mixed Emulsion of Testes and Prostate.

The details of these experiments on rabbits Nos. 17 and 38 are shown in Table I (Exps. 4, 6 and 8). The results of these experiments agree well with those of the two previous groups as is shown clearly in Table III.

The increase in nitrogen metabolism produced in rabbits with thyroids after the injection of prostate alone or of prostate and testis simultaneously was not found in the thyroidectomised rabbits. Moreover, when an emulsion of both prostate and testis was injected into the latter animals, on an average, a slight decrease was observed in the nitrogen metabolism, *i.e.* a change typical of testicular emulsion.

Therefore these groups of experiments show, firstly, that in order to produce an increase in the nitrogen metabolism after injection of prostate emulsion the thyroid gland must be present; secondly, that in thyroidectomised animals, when an emulsion of both testis and prostate is injected, the influence of the former gland prevails. The latter result is also corroborated by the decreased urinary flow in Exps. 4, 6 and 8, *i.e.* a change in the excretion of urine typical of the influence of testicular and not of prostate emulsion was observed. No definite or constant change was obtained in the percentage of nitrogen excreted in the faeces.

A slight increase in weight of rabbit No. 17 was observed during the injection period and the following control periods, but rabbit No. 38 slightly decreased in weight in the control periods following the injections.

Injections of Insulin-like Extracts (B *and* C) *of Bull's Testes.*

Extract B was injected into rabbits Nos. 14, 17, 26, 31 and 41 and details are given in Table I (Exp. 7) and Table II (Exps. 11, 12, 16, 17 and 18). Extract C was injected into rabbits 23 and 33 (see Table I (Exp. 2) and Table II (Exps. 14 and 15)). The difference between the influence produced by extracts B and C upon nitrogen metabolism was only of a quantitative nature and was due to the presence of a greater amount of testicular insulin in extract B than in C (see the influence of these extracts upon the blood-sugar of rabbits mentioned above). When 0·8–1·5 mg. of extract B or 1·2–2·3 mg. of extract C per kg. and diem were injected subcutaneously into normal or castrated rabbits, in nearly all cases, only a slight fall was produced in the nitrogen metabolism. Similar or even smaller doses injected into thyroidectomised rabbits produced a much more pronounced decrease in the nitrogen metabolism (see Table III). This effect lasted and, in some cases, was only present or most pronounced in the control periods following the injections.

The percentage of nitrogen in the faeces was slightly increased in about half of the experiments and was decreased or not altered in the other half.

Urinary flow was quite definitely decreased in all cases during the control periods (5–8 days) following the injections, but during the injection period itself it was increased in five cases (36 %) and decreased in nine cases (64 %).

The weights of the animals under the influence of the injections changed a little in both directions; for example, during the control period immediately following the injections half increased and half decreased in weight. Therefore it was quite impossible to establish any relationship between the change in weight and the change in urinary flow, which, as we mentioned above, was diminished in all animals during this same period.

Discussion.

The presence or absence of the thyroid gland seems to be of importance for the changes in the nitrogen metabolism produced in rabbits by emulsions or extracts of testis and prostate glands. Testis emulsion and insulin-like substances obtained from the testis produced only a slight fall in the nitrogen metabolism of normal or castrated animals, probably because the thyroid hormones counteract this influence. In a previous paper [Korenchevsky, 1914] it was shown definitely that the thyroid gland is a great stimulant to nitrogen metabolism. Therefore it is quite reasonable to suppose that, as this effect was lacking in the thyroidectomised animals, the fall in the nitrogen metabolism after injections of testis emulsion and testicular insulin-like substances would be much greater than in the animals with thyroids. These observations agree well with the experiments of Bodansky [1923], Houssay and Busso [1924] and Ducheneau [1924], who found that toxic effects and lowering of blood-sugar produced by insulin were more pronounced in thyroidectomised than in normal animals.

The similarity between the influence of testis emulsion and that of insulin upon nitrogen metabolism and to some extent upon the excretion of urine suggests that the fall in nitrogen metabolism and urinary flow obtained in rabbits after the injection of testis emulsion might be partly due to the presence of insulin-like substances in the testes. The same explanation might apply to the decrease in the nitrogen metabolism of rabbits after the injection of kidney emulsion [Korenchevsky and Carr, 1925].

Experiments with prostate emulsion injected into normal and thyroidectomised rabbits have shown that the thyroid gland must be present in order to obtain an increase in the nitrogen metabolism: in thyroidectomised rabbits an emulsion of prostate alone produced practically no change in the nitrogen metabolism, whereas an emulsion of both prostate and testis usually decreased the nitrogen metabolism, although to a less degree than when an emulsion of testis alone was injected.

It seems probable, therefore, that the prostate emulsion produced its influence upon the nitrogen metabolism of rabbits indirectly through the thyroid gland. This mechanism is not found to work with the testicular emulsion, which, as the experiments on dogs show [Korenchevsky, 1925, 2], increases the nitrogen metabolism of thyroidectomised as well as that of normal and castrated animals.

Unfortunately it is not possible from our experiments to explain the mechanism which decreases urinary flow after the injections of testis emulsion and testicular insulin-like substances.

In our experiments thyroidectomy did not influence the changes in the excretion of urine produced by testis emulsion or insulin-like substances, but it did affect the change in flow after injections of prostate emulsion.

CONCLUSIONS.

1. An homologous emulsion of testis decreased the nitrogen metabolism of thyroidectomised rabbits much more than that of normal or castrated animals.

2. This emulsion also decreased urinary flow to about the same degree in rabbits both with and without thyroid glands.

3. The insulin-like substances isolated from testes and injected into thyroidectomised rabbits also produced a more pronounced decrease in nitrogen metabolism than in rabbits with thyroids.

4. These substances decreased the excretion of urine in both thyroidectomised and non-thyroidectomised animals during the periods following the period of injection, as did testicular emulsion. During the period of injection itself the flow of urine increased in about one-third of the experiments and decreased in the other two-thirds.

5. Thyroid hormones appear to counterbalance the depressing influence on N metabolism of rabbits produced by an emulsion of rabbit's testis or of testicular insulin-like substances.

6. The similarity between the effects of testicular emulsion and of the insulin-like substances extracted from testis suggests that in the former case the fall in nitrogen metabolism, and possibly in urinary flow, is in part due to the presence of insulin-like substances in testes.

7. The same explanation might apply to the decrease in the nitrogen metabolism of rabbits after an injection of kidney emulsion since insulin-like substances have also been isolated from kidney.

8. An homologous emulsion of prostate alone produced practically no change in the nitrogen metabolism of thyroidectomised rabbits, whereas in that of rabbits with thyroid glands a definite increase was observed.

9. This latter increase was also absent when an homologous emulsion of both prostate and testis was injected into thyroidectomised rabbits; instead, on an average, a slight decrease was observed, indicating the predominance of the testicular part of this mixed emulsion.

10. Prostate emulsion appears to increase nitrogen metabolism of rabbits by stimulating the activity of the thyroid gland.

11. Prostate emulsion increased the flow of urine in the thyroidectomised rabbits, whereas, on an average, the excretion of urine was decreased in rabbits with thyroid glands.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We also wish to express our sincere gratitude to Prof. C. J. Martin for his continuous support and criticism of this work.

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CXX. A LABORATORY APPARATUS FOR THE WET GRINDING OF PLANT TISSUES OUT OF CONTACT WITH AIR.

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(*Received August 22nd, 1925.*)

In extracting the proteins from potato tubers great difficulty is experienced on account of the action of oxygen on tyrosine, causing in the early stages of the experiment the formation of melanin, the removal of which is difficult once it has been formed. An apparatus has therefore been devised in which the tubers may be finely pulped and the pulp filtered in the absence of oxygen, the exclusion of the latter being sufficiently thorough for the filtered juice to be of only a faint blue colour[1] instead of the intense black colour resulting when oxygen is present.

Since the yield of protein is likely to be proportional to the number of cells of which the walls are broken, a high degree of efficiency of grinding was also desirable. It was secured by means of a rapidly rotating alundum wheel tearing apart the tissues of the tuber. Turgid potato tubers have, in fact, been pulped with the machine at a rate of about 1 kg. per hour sufficiently finely to rupture almost every cell wall, without, however, breaking the starch granules.

Since the machine seems capable of application to many biochemical problems a brief description and diagram are given below in the hope that it may be of use to other workers.

An inverted glass bell-jar *A*, which serves as a receiver for the pulp, is suspended by its rim through a circular hole in a table *B*, a ring of rubber, *C*, acting as a cushion between the two. The bell-jar is held securely in position by the brass plate *D*, which is firmly attached to the table by means of three bolts and thumb screws *F* (one only shown in the diagram). A second ring of rubber *E*, placed between the brass plate and the bell-jar, serves to make an air-tight joint between them. Passing through the centre of the plate *D*, is a shaft and bearing *H*, which carry the alundum grinding wheel, *G*, on the lower end, and at the upper end can be connected by a leather band to a $\frac{1}{4}$ H.P. motor.

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[1] This blue colour forms the subject of a note published in the *Annals of Botany*.

The potatoes to be ground up are inserted through a cylindrical brass tube *I*, which is fitted tightly into a hole in the brass plate *D* by means of screws, so that its basal end almost touches the grinding wheel. The tube is made in sections which may be screwed together so that its length may be altered at will. A second but much shorter brass tube of the same diameter, *J*, is soldered into a second hole in the plate lying just to the side of the first: this, for the sake of convenience in drawing, is shown out of position in the diagram. A piece of strong rubber tubing *K* (the inner tube of a small motor-bicycle is of a convenient size and curvature for the purpose) is attached to the brass tube *J*, and is closed at its free end with a block of wood *L*, into which is fitted the handle of a steel wire brush *M*. The rubber tube is sufficiently long for the brush, in a position of rest, to be just free of the grinding wheel, but it allows sufficient freedom of movement to permit the brush to be brought down on the surface of the rotating wheel to clean it without either discontinuing the grinding or risking the introduction of oxygen. The three parts of the apparatus are connected up together, by means of glass and rubber as shown in the diagram, in such a way that the whole of the air in the apparatus can be replaced by an inert gas, not lighter than air, which enters under slight pressure by the inlet tube *Q*, passes in the directions indicated in the diagram by arrows, and finally emerges from the top of the tube *I*. In the case under consideration carbon dioxide was used to displace the air, though it is probable

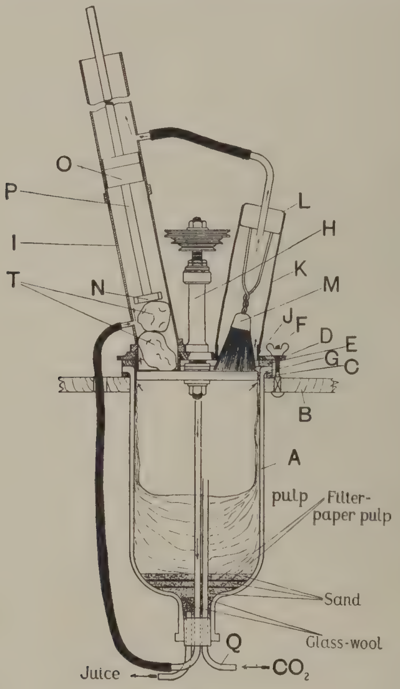

Fig. 1.

that nitrogen might have served equally well. During an experiment a continuous stream of gas is kept passing through the apparatus in order to displace any air that may be carried in when the potatoes are introduced into the tube *I*. The tubers are lowered slowly, one at a time, to the base of the tube *I* on the end of a long skewer with a corkscrew end, which may be disengaged from the potato by unscrewing. Not more than two tubers at a time are put in the tube, otherwise there is a risk of jamming. They are pressed down against the grinding wheel by means of a plunger, the end *N* of which is about half the area of the cross section of the tube; this is sufficiently large to prevent the slipping of the end past a tuber and small enough to cause no serious introduction of oxygen when the plunger is pushed into the tube. A cylindrical piece of lead *O*, is screwed on to the rod *P*, of the plunger, about

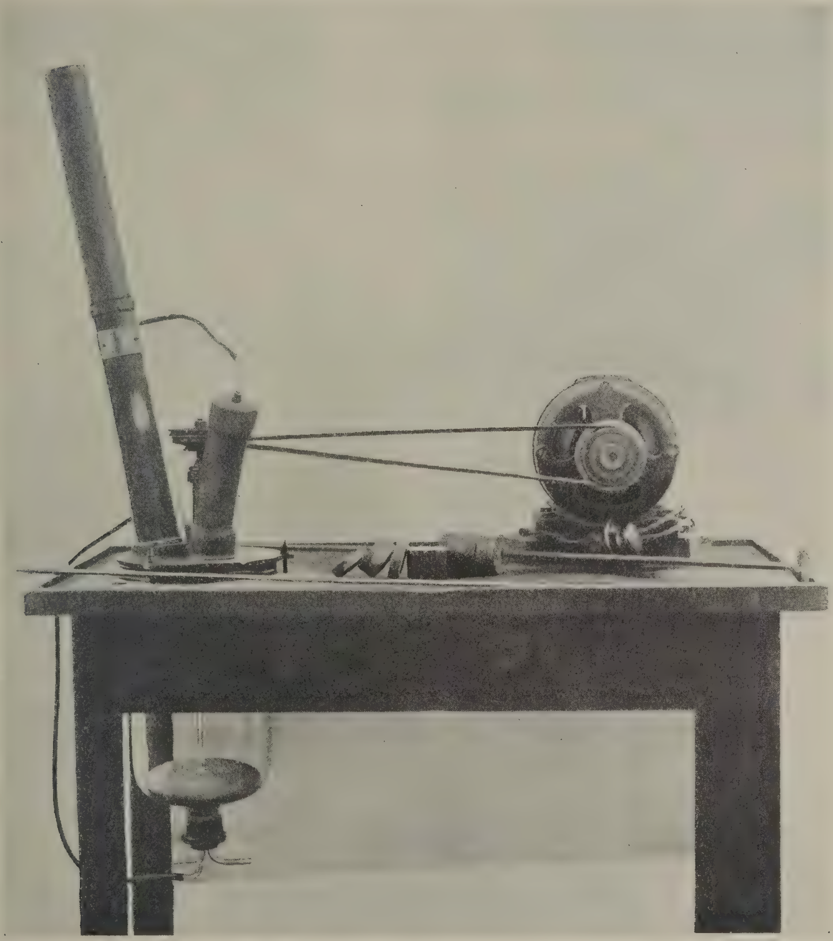

Fig. 2.

half-way up its length to weight it: additional perforated leaden cylinders may be slipped over the rod to increase the pressure on the tubers. The wheel is spun at about 1500 revolutions per minute by means of a $\frac{1}{4}$ H.P. electric motor. The resulting pulp is quite white and is sufficiently liquid to run down the sides of the bell-jar, if turgid tubers are used, without the addition of any water.

As stated above, this material can be pulped at a rate of 1 kg. per hour. When the pulp is examined under a microscope it is seen that every cell has been broken except occasional "stone cells" and, in the case of each tuber, the last thin piece of skin which passes between the base of the tube *I* and the wheel; by adjusting the parts carefully the weight of these unground pieces can be rendered negligible compared with the total weight of the tubers used.

When flaccid tubers are to be ground it is necessary to add a little water. Provision has been made for this by means of a narrow brass tube, passing through the plate *D*, close to the spindle and to the tube *I*, through which water or filtered juice may be dropped so as to fall on the rotating wheel and be flung on the material being pulped. This tube is completely plugged up when not actually in use to prevent the formation of a small air-pocket.

The pulp collecting in the bell-jar is filtered by means of alternate layers of filter paper pulp and sand, two layers of the former and three of the latter, supported on a plug of glass wool as shown in the diagram. This gives a filtrate which is only very slightly turbid.

It is found necessary to bubble the carbon dioxide through ether before it enters the bell-jar. In this way the foam into which the wheel "whips" the pulp is discharged.

If the residual pulp be shaken with water and allowed to settle it is possible to separate mechanically:

(1) cell-wall material which floats away readily;

(2) starch grains;

(3) any unground pieces of skin.

These facts suggest that the machine might be used for the separation of cell-wall material for its chemical examination, and that by its means the starch content of potato tubers and similar material might be estimated by a simple mechanical separation.

The apparatus was constructed from the writer's design by Messrs F. and T. Mercer, Apparatus Makers, St Albans, to whom he wishes to express his best thanks for the care and skill exercised by them in its construction.

CXXI. GLUTATHIONE.

ITS INFLUENCE IN THE OXIDATION OF FATS AND PROTEINS.

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I. Introductory.

This paper gives an account of observations which illustrate certain of the properties of glutathione as a promoter of oxidations. The fresh facts made available are as yet quite insufficient to explain its precise functions in living tissues, but they carry suggestions for future work. The experimental results touch at some points on phenomena concerning which basal chemical knowledge seems lacking. They cannot therefore be succinctly described[1].

Meyerhof has shown that the oxidation of certain unsaturated fatty acids is promoted by the presence of thiol compounds, and the results he obtained by the use of thioglycollic acid and cysteine (in particular the former) are easily reproduced with the dipeptide, though with certain differences that are significant. It will be shown in this paper that a curious limited oxidation of proteins is brought about by the agency of glutathione, exerted in quite peculiar relations. The observations of Miss Thurlow [1925] carried out in this laboratory have shown that in the progress of the oxidation of the SH group peroxide formation occurs, and that under such circumstances, in the presence of a peroxidase, certain secondary oxidations may be induced. On these lines it is probable that the functions of glutathione may come into relation with the events of carbohydrate metabolism.

In the experiments to be described, in so far as they have involved the use of tissue preparations, muscle tissue has been almost exclusively employed. In certain details, though not in fundamentals, the relations of muscle fibres with glutathione are special. The behaviour of cellular tissues requires separate treatment.

Since the publication of the paper by Dixon and myself [1922], which followed the original account of its isolation [Hopkins, 1921], the constitution of glutathione as diglutaminyl-cysteine has been established on what seems to be conclusive evidence by the work of Quastel, Stewart and Tunnicliffe [1923], and by its synthesis in the hands of the latter two investigators [Stewart and Tunnicliffe, 1925]. The natural product has now been many times prepared (chiefly from yeast) by different individuals, using the method originally described. Szent-Györgyi [1925] has endeavoured to modify that method, but states that he was unable to improve upon it. With experience the original yields have been increased. Some 20 g. of the pure substance from 50 kg. of moist pressed yeast are obtained without difficulty. This falls much below the maximal figure given by direct titration, *circa* 0·2 % [Tunnicliffe, 1925]; but it is certain that serious losses occur during the process of isolation[1]. Tunnicliffe [1925] finds that muscle contains about 0·035 % of which about one-third only has been actually isolated. It is in any case sure that Abderhalden and Wertheimer [1923] are mistaken in supposing that the concentration of free cysteine in the tissues is at all comparable with that of the dipeptide. I possess some evidence to show that the former amounts at the very most to one-twentieth of the latter and is probably much less. Free cysteine cannot play a part of quantitative importance in promoting tissue oxidation.

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[1] Many of the observations described were made more than two years ago, but publication was unavoidably delayed. Unfortunately, more recent work—much limited by lack of leisure—still leaves the subject in a position which is curious and obscure. I think the fresh facts described are of real interest, however, and publication at the present time may stimulate the work of others. The paper should be looked upon, in part at least, as a prolegomenon to publications from this laboratory which will appear in the near future.

Any description of the properties of glutathione and any study of its functions must of course take due account of the important observation of Warburg and Sakuma [1923], showing that the SH group of thiol compounds is not strictly autoxidisable, but is oxidised by molecular oxygen only in the presence of minute traces of iron. That this holds in the case of glutathione (or, at least, that the autoxidation of its thiol form is relatively very slow in the absence of all but irremovable traces of iron) has been shown by Harrison [1924]. It would be desirable, therefore, to carry out all experiments with preparations containing a minimal, but exactly known, quantity of the metal. Owing however to the laborious nature of the process required for its complete removal and the waste of highly expensive material involved, either in the removal of the traces present in the preparations used, or in estimating these traces, this course is scarcely possible in practice. Since Warburg's discovery the use of specially pure reagents has yielded preparations containing, at most, infinitesimal quantities of the metal. The employment of these, instead of earlier, preparations has made surprisingly little difference to the experimental results. In any case, this relation with iron does not remove any significance from the presumed functions of the thiol group in respiration. The metal is only another link in the chain. None of the results to which significance is attached in this paper is due to the direct influence of iron alone. This question receives frequent reference; but, otherwise, when the addition of glutathione to a system is mentioned, the presence of such minute amounts of iron as the preparation might contain is presumed.

A statement has been made by Szent-Györgyi [1925] which, if correct, could not fail to affect profoundly any views concerning the biological functions of glutathione. This author states that the thiol group as it exists in the tissues (where it is certainly in contact with sufficient iron) is not oxidisable. He prepared an extract from the liver of a guinea-pig by grinding up the organ with specially prepared distilled water. The extract was boiled and filtered. The filtrate gave a strong nitroprusside reaction which did not (apparently) diminish on aeration. He does not mention the p_H of the preparation, and he aerated it for 15 minutes only. But such rapid oxidation is certainly not to be expected under such conditions. It would indeed be disconcerting to find that any factor supposed to be concerned with respiratory oxidations could so rapidly disappear from a tissue preparation of the kind. I have many times seen the nitroprusside reaction completely disappear from unwashed liver tissue suspended in distilled water at p_H 7·4 when shaken in a Barcroft apparatus, but only after 5–6 hours at 37°. For special purposes I have many times proceeded as follows. The liver (or other organ) after grinding (the pulp being first squeezed through coarse muslin) is mixed with twice its volume of distilled water, the mixture made neutral or slightly alkaline, placed in a large stoppered bottle and shaken vigorously in a mechanical shaker. I have several records showing that under these conditions the original intense nitroprusside reaction of an aseptic liver preparation may completely disappear within 8 hours at room temperature. When the preparation is first boiled and filtered the oxidation in the filtrate seems to be a little slower, but with efficient aeration the nitroprusside reaction always finally disappears. Since the publication of the above author Mr Albert Carruthers has repeated this procedure using, instead of ordinary distilled water, water twice distilled in quartz apparatus. This made no difference to the result.

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[1] It is undesirable to attempt to isolate the substance from less than 20 kg. of moist yeast.

In the discussions which follow, in order to save space and printing, the reduced, or thiol form of glutathione is generally referred to as GSH, and its disulphide form as G_2S_2. It is often convenient to speak of "SH" and "S-S" as though they were independent chemical entities.

II. EXPERIMENTAL METHODS.

All the estimations of oxygen uptake were made in the Barcroft "differential manometer" apparatus of the type previously described in publications from this laboratory. The flasks are flat bottomed, shaped so as to provide a large surface for the suspension fluid and secure efficient aeration when the instrument is shaken. It was easy to observe that in none of the experiments described did the rate of oxygen supply become a limiting factor. The instruments were carefully calibrated and four could be shaken side by side by the same motor while immersed in the same bath. Different systems could therefore be compared under identical conditions. Unless otherwise stated, all the measurements were made at 37°. For a reason mentioned in the next section, the phosphate buffer solutions used as suspension fluids in earlier experiments [Hopkins and Dixon, 1922] were given up. As in the great majority of cases the oxidations studied involved no change in p_H, Ringer solution or distilled water was used. It was found quite easy under these circumstances to adjust the p_H of the systems studied and in the experiments described it was found to be unchanged at the end. The dipeptide (partially or completely neutralised with soda) itself provides slight buffer action.

Many of the results are given in the form of curves relating the oxygen uptake with time. Meyerhof [1923] has correctly remarked that when tissue preparations are shaken in apparatus like the Barcroft manometer, the absolute velocity of uptake may be so greatly affected by accidental factors, such as the physical condition of the preparation, that its measurement has little significance. In certain cases it is doubtless of small value. Nevertheless, when relative, and not absolute, velocities are in question, and in some other cases, curves may give desirable information which is not supplied by a statement of total uptake. When oxidising systems are shaken side by side and when one factor only is varied as between a particular system and a control system, a comparison of the curves obtained is often of value. Those given in the figures are quite unsmoothened.

III. THE OXYGEN UPTAKE (WITH GLUTATHIONE) OF WASHED MUSCLE AND ITS THERMOSTABLE RESIDUE.

In the paper by Hopkins and Dixon [1922] it was shown that when washed muscle is extracted with boiling water, washed with alcohol, dried *in vacuo* and powdered, the dry powder, called in the above paper the thermostable residue, though showing no oxygen uptake by itself, displays a definite uptake in the presence of glutathione. A corresponding amount of the original washed muscle without further treatment is itself capable of the same quantitative uptake, but to establish a sufficiently active system within the swollen and somewhat impermeable fibres of the washed preparation a relatively high concentration of glutathione in the external fluid is necessary. A concentration of the order $M/200$ secures a brisk uptake with the powder. In the case of the washed muscle the uptake with $M/40$ is still relatively slow, but it reaches the same level as that of the powder within a reasonable experimental period (Table I).

Hopkins and Dixon [1922] using Clarke and Lub's phosphate buffer solutions obtained an oxygen uptake of about 400 mm^3. per g. of dry powder. It was shortly afterwards found that the high concentrations of phosphate used limit the total oxygen to a remarkable extent, for reasons not yet fully explained. The uptake of an average preparation in Ringer's solution at p_H 7·6 is of the order of 2000 mm^3. per g. A great number of such estimations were made at a time when more importance seemed to belong to the actual figures than is really the case. Unselected instances are given in Table I.

Table I.

Oxygen uptake of muscle preparations suspended in aqueous solutions (3 cc.) of glutathione (disulphide). Except where otherwise stated all preparations were in the form of the dry thermostable powder. p_H of system 7·6 in each case.

No. of Exp.	Species of muscle	Weight employed g.	Concentration of G_2S_2	Time of observation hours	O_2 uptake mm.^{3}	Uptake mm.^{3} per g.	Remarks
1	Cat	0·2	$M/200$	7½	385	1925	
2	Dog (1)	0·2	$M/200$	7½	395	1975	
3	Dog (2)	0·2	$M/200$	8	400	2000	
4	Rabbit	0·2	$M/200$	7	363	1815	
5	Rabbit	1·0	$M/50$	8	285	1425 (on dry weight)	Moist washed muscle—uptake not complete
6	Rat	0·2	$M/200$	7½	454	2270	
7	Frog	0·2	$M/200$	7	310	1550	
8	Frog	0·5	$M/50$	8	110	1100 (on dry weight)	Moist washed muscle—uptake not complete

It was shown in a previous paper that when the dipeptide forms an oxidising system with washed muscle it suffers itself no oxidation save the reversible change in which the sulphur group is alone concerned. The observations in question involved the use of phosphate buffers. It seemed worth while to demonstrate the fact again with the non-buffered media in which the uptake of the system is so much higher. The curves of Fig. 1 show that the total oxygen taken up reaches practically the same value, though of course at different velocities, with greatly varying concentrations of glutathione. These curves were obtained with the muscle powder; but since, as stated above, muscle when simply washed gives with high concentrations of glutathione the same uptake as a corresponding amount of powder with a lower concentration, it is sure that the oxidising agent in washed muscle which oxidises

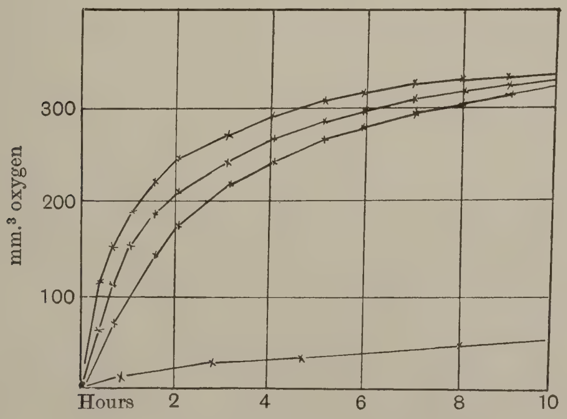

Fig. 1. Oxygen uptake of thermostable powder from cat's muscle, 0·2 g. in 3 cc. Ringer at p_H 7·6. Of the three upper curves the lowest shows the effect of 4 mg., the middle of 8 mg., and the highest of 16 mg. glutathione (G_2S_2). The bottom curve shows the uptake of the powder alone.

free glutaminic acid [Thunberg, 1920] does not affect the glutaminyl moiety of the dipeptide.

Meyerhof [1923], in the important communication which appeared shortly after that of Dixon and myself, also describes observations upon the thermostable residue ("Alkoholpulver"). He reports uptakes (with thioglycollic acid) as high as 5000 mm.^{3} per g., though many of his data point to a lower figure than this. A comparison of his figures with my own is of small significance, however. Many of them were obtained under different conditions, especially in respect of the hydrogen ion concentration in the systems studied. Variations in this affect more than velocity of uptake. It will be shown, for instance, that in acid systems the oxidation of fat is favoured, whilst oxidation of quite other material proceeds best at the neutral point or in slightly alkaline solutions. A difference in hydrogen ion concentration will therefore affect the result both quantitatively and qualitatively (see Figs. 9, 10 and 11, p. 813, section VI). Meyerhof used thioglycollic acid. In the experiments of Table I glutathione was employed not as the thiol compound, but in its disulphide form.

As a matter of fact, the significance of the oxygen uptake of the thermostable residue is somewhat different from what may have been suggested or implied in previous publications. It will be convenient, before returning to it, first to consider the behaviour of systems containing unsaturated fats and glutathione alone, and afterwards that of fat-free tissue-systems. The former take up oxygen only when the thiol form of the dipeptide is present from the first, since the fats do not reduce the disulphide form. Oxidation in the latter proceeds with either form.

IV. The Oxidation of Unsaturated Fatty Acids and Fats.

In aqueous systems containing GSH together with an emulsion of unsaturated fatty acids (linolenic, linoleic), so long as the p_H is of the order of 3·0 and 4·0, the concentration of the SH group is long (though not indefinitely) maintained during the aeration of the system. Oxygen many times in excess of what is required to oxidise the SH to S-S is taken up and a corresponding amount of fatty acid oxidised. When, however, the hydrogen ion concentration approaches the neutral point, or when it is near to this on the alkaline side (p_H 7·4–7·6), the behaviour of such systems is profoundly changed. The SH group (the presence of traces of iron being presupposed) is then so freely oxidisable that, unless its concentration in the system be relatively high, it rapidly disappears, even in the presence of the fatty acid. The system then becomes inert before more than relatively small amounts of fatty acid have been oxidised. The behaviour of systems composed of GSH and unsaturated fatty acids seems to be therefore essentially the same as that described by Meyerhof for the systems lecithin-thioglycollic acid and linolenic acid-thioglycollic acid. (See Table II and compare the results of Exps. 1–5 with those of Meyerhof [1923, Table XV, p. 548].)

In considering acid systems in which the concentration of the SH group falls off very slowly during the aeration of the system, the view of Meyerhof [1923, p. 561] as to the mechanism of the oxidation seems adequate. It will be remembered that he postulates the preliminary formation of a peroxide by two thiol groups. This peroxide is assumed to decompose then in such a way that the oxygen is wholly transferred to the unsaturated linkages of the fatty acid, whilst the original SH groups are reconstituted. The oxidation of the former is what would usually be considered as a catalysed reaction and the thiol group is concerned essentially with oxygen transport.

Table II.

Ref. no.	Material employed 10 mg. as emulsion in distilled water	p_{H}	Time for uptake hours	Total uptake mm.^{3}	O_2 equivalent of SH present mm.^{3}	O_2 to fat mm.^{3}	Remarks
1	Linolenic acid	3·5	6	210	32	178	Acid systems
2	,,	3·8	6	225	41	184	In all cases the up-
3	Linoleic acid	3·5	6	140	32	108	take was still pro-
4	Mixed acids from linseed oil	3·2	5½	710	200	510	gressing at end of observation
5	Mixed acids from linseed oil	3·5	8½	550	90	460	
6	Linolenic acid	7·6	6½	40	26	14	Neutral or alkaline
7	Mixed acids	7·4	7	62	35	27	systems
8	,,	7·6	7	830	425	405	
9	,,	7·6	4	180	90	90	
10	,,	7·6	3	325	165	160	Uptake complete
11	Linoleic acid	7·4	5	250	132	118	in all cases
12	Lecithin	7·6	6½	732	390	342	

It is important, however, to consider in greater detail the behaviour of the system GSH + unsaturated fatty acid at p_{H} 7·4–7·6. Meyerhof states that at the neutral point the oxidation of fat under the influence of thioglycollic acid is completely inhibited. With reduced glutathione this is not the case. I have found as the result of a number of observations that within a wide range of SH concentrations the oxygen uptake of the fatty acid, or its sodium salt, is equal, or approximately equal, to the amount of oxygen required to oxidise the SH. In other words, when the reaction ceases, the total uptake proves to be twice the oxygen equivalent of the SH present. To show this by the use of the Barcroft apparatus, it is necessary to work with relatively large concentrations of GSH. If small amounts are used, such as are ample for the demonstration of the more strictly catalytic effect at p_{H} 3·5, the total uptake at 7·6 becomes too small for accurate measurement, though the relation in question may nevertheless be indicated (Table II, Exps. 6 and 7). Moreover, unless the equilibration of the instrument at the beginning of the experiment is carried out with the flasks filled with nitrogen (oxygen being admitted only after equilibration), some oxidation of the SH group may occur before the system is, so to speak, properly established. This error does not count for much when the total amount of glutathione is sufficiently large. The figures of Exps. 6–12 of Table II show that the relation mentioned above holds within the limits of experimental error at p_{H} 7·4–7·6. It is the familiar relation found so often when the autoxidation of one molecule induces the oxidation of a second non-autoxidisable molecule. The SH group is, in neutral or slightly alkaline systems, no longer an oxygen carrier in the sense that Meyerhof postulates for acid systems. It would seem as though its case becomes one represented by the first of the alternative schemata which have been so familiar from the publications of Engler and his colleagues; *A* being an Autoxidator, and *B* an Acceptor.

$$(1)\ AO_2 + B \longrightarrow AO + BO; \qquad (2)\ AO_2 + 2B \longrightarrow A + 2BO.$$

The first is an induced or coupled oxidation; the second is a catalysed oxidation. Whether it be justifiable or not to claim that the change in hydrogen ion concentration actually changes the type of reaction from (2) to (1), there is no doubt that in neutral or slightly alkaline solutions the oxidation of the SH group induces a limited oxidation of the unsaturated fatty acids, and, at the same time the figures obtained strongly suggest that equipartition of oxygen actually occurs. Such cases are at any rate common. The simple schema (1) above may not, of course, properly represent the details of the reaction. Without knowledge, however, as to what happens at the unsaturated linkages of the fatty acids as the result of the limited oxidation in question (it is not necessarily the same as what occurs in more acid systems), it is impossible to say whether or not there are chemical grounds against the above assumption.

It is at any rate of some interest to know that the oxidation of fats is influenced by glutathione at hydrogen ion concentrations near to that of the tissues as well as at others more remote.

The figures of Table II and the curves of Figs. 2 and 3 show plainly the effect of change of p_H on systems comprising GSH and fatty acids.

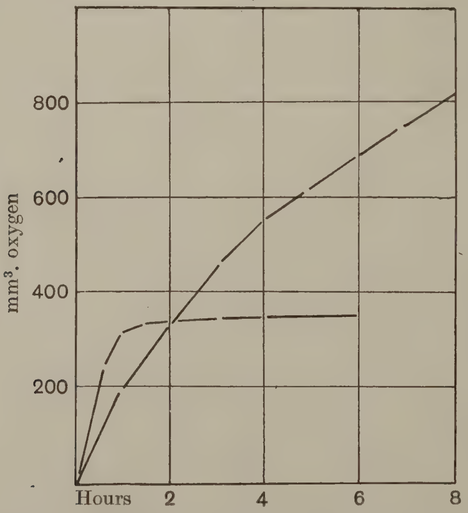

Fig. 2. Showing effect of p_H upon the oxygen uptake of mixed fatty acids from linseed oil in presence of GSH. Prolonged uptake at 3·8; short uptake at 7·6. Acids 10 mg. in emulsion. Oxygen equivalent of SH present 182 mm³. The fatty acids by themselves showed no uptake

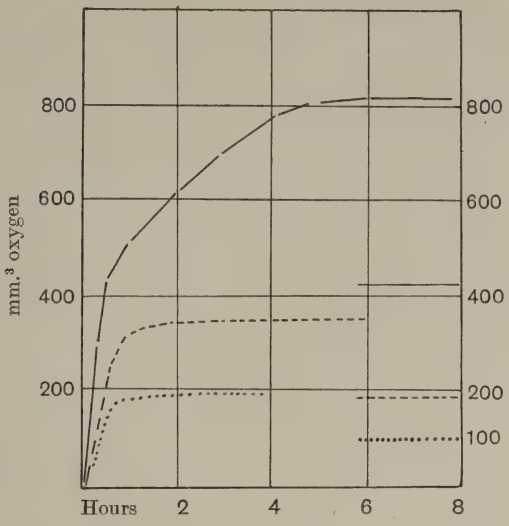

Fig. 3. Oxygen uptake of fatty acids with varying amounts of GSH at p_H 7·6. The horizontal lines mark the oxygen equivalent of the SH present in each case.

It is perhaps desirable to refer here to a point in the technique used for these experiments which might seem to make it inadequate. When, in any combination, fat emulsions are shaken with air, the degree of dispersion of the fat phase might be expected to prove a factor in the velocity of oxygen uptake so dominant as to obscure the effect of other variants. This is not the case. In practice it has proved easy to reproduce any given experimental result, including even a velocity curve, without any special effort to standardise the emulsion, save in respect of the amount of fat present in it and the p_H of the system. Once the preparation is measured into the flask of the Barcroft apparatus, the shaking of the instrument maintains dispersion of the fat, and when other conditions have been kept constant, it has proved easy to obtain accurate reproductions of a given result on successive occasions, without giving more than ordinary care to the rate of shaking. When in the experiments described emulsions at p_H 7·4–7·6 were under study, small quantities of sodium carbonate were used to adjust the reaction and sodium salts were therefore present from the first. In this case also smooth and reproducible curves of uptake were obtained. It is remarkable that in such systems the surface relations should appear to play a relatively minor part in determining the velocity of oxidation.

It is clear that the significance of the results so far described is in any case quite independent of the absolute velocity of oxygen uptake. The curves of Figs. 2 and 3 satisfactorily illustrate however the complete change of type which the oxidising system undergoes on change of p_H.

In observations dealing with the oxidation of linseed oil emulsions, to which reference will be made immediately, changes of velocity during the course of uptake are assumed to be of chemical significance. I could find indeed no evidence whatever to suggest that changes in the grade of dispersion or other non-significant factors played any part in them.

In parenthesis, it may be here remarked that although minute traces of iron (such as were doubtless present in the preparations used) may be necessary for the autoxidation of the SH group of glutathione, none of the results described is brought about by the direct effects of the iron as such. If (as I have repeatedly found) any previously active preparation of the reduced dipeptide be oxidised by aeration to the disulphide form, it then induces no observable oxygen uptake by the fatty acids, although of course its iron content is intact. An ionised salt of iron will doubtless in weakly acid systems catalyse the oxidation of linolenic acid at concentrations which, in an absolute sense, are low; but to produce an observable effect the concentration must be much larger than that introduced with the glutathione into the systems studied. At p_H 7·4–7·6 iron by itself fails to promote oxidation of the fatty acids, or their alkali salts, and the point does not arise.

It is a remarkable fact that the course of oxygen uptake by the glycerides of linoleic and linolenic acids, under the influence of GSH, differs in several particulars from that exhibited by the free fatty acids or their sodium salts. This statement is based upon experiments done with the purest obtainable specimens of linseed oil.

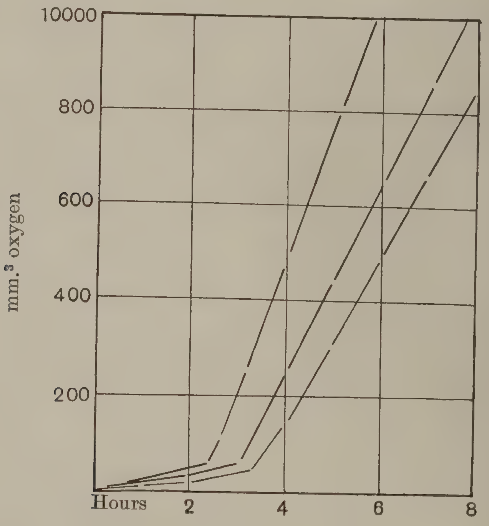

Fig. 4. Uptake of linseed oil (20 mg. in emulsion) with GSH at p_H 3·5. Left-hand curve with 12 g., middle curve with 8 mg. GSH. Right-hand curve 8 mg., but with oil first boiled *in vacuo*.

In acid systems (p_H 3·5–4·0) the curve of uptake of the system (GSH + linseed oil emulsion) shows a marked "period of induction," during which the system is almost completely inert. This period generally lasts from 1–3 hours, being diminished, but never abolished, by increasing the concentration of GSH. It is diminished by previous aeration of the oil, and lengthened if the oil be first boiled *in vacuo* (see curves of Fig. 4). These phenomena are of course familiar from studies concerned with inorganic "driers," or "siccatives," used technically to accelerate the drying of linseed oil in paints and varnishes. The fact that the progress of oxygen uptake during the drying of the oil yields the sinuous time curve (with its period of induction) characteristic of an autocatalytic reaction has been established by many observers. Evidence suggests that the autocatalyst involved is an autogenous peroxide which when the fat is aerated alone only very slowly reaches an effective concentration. Rideal [1919], Coffey [1921], and others have shown that the period of induction persists, though much shortened, in the presence of driers. Agents which promote the oxidation of linseed oil may therefore be "pseudo-catalysts" serving to assist in the formation of (or in maintaining the concentration of) the autocatalytic peroxide. The curves of Fig. 4 obtained at p_H 3·5 suggest that at least when glycerides are concerned the thiol compounds come into the same category[1]. I have never succeeded in obtaining evidence of an induction period in the case of the free fatty acids, however treated, or at any p_H. If Meyerhof's view, as stated above, concerning the oxidation of the free acids by SH compounds be correct, and in acid systems it seems fully justified, it would seem that the mechanism of oxidation is actually different in the case of the fatty acids, or their sodium salts, and that of their glycerides, respectively. I cannot find from the chemical literature that any study of the effect of ordinary driers upon the free acids has been made[2].

At p_H 7·4–7·6 linseed oil again shows a behaviour which is strikingly different from that of free linoleic and linolenic acids.

When an emulsion of the oil is shaken in the presence of relatively high concentrations ($M/100$–$M/50$) of GSH, in a neutral or weakly alkaline medium, the equipartition of oxygen so characteristic of systems containing the free fatty acids is not seen. The curve of oxygen uptake assumes a characteristic form, which has shown itself constant in a great number of observations (Fig. 5). There is no period of induction. The high initial velocity falls off somewhat rapidly and gives place to an uptake with a lower velocity which remains exactly linear for several hours and until the fat is nearly all oxidised. The inflexion in the curve occurs when sufficient oxygen has been absorbed to correspond nearly (but not quite) with that required to oxidise the SH present. During the linear period the concentration of SH is therefore very low, a circumstance which has been repeatedly confirmed by the use of the nitroprusside reaction. It would seem that during the first stage of uptake the SH group of the glutathione is oxidised without any associated effect upon the fat. The impression is nevertheless given (though it is not necessarily correct) that an efficient concentration of the thiol group must survive long enough to secure the establishment of some condition in the system which is not found initially. When quite small concentrations (less, say, than $M/200$)

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[1] If so their influence upon the unsaturated linkages must be somewhat different from that exerted when they are oxidising fatty acids. In spite of the general assumption it is not proven that the autocatalyst is necessarily a peroxide. The following observation has some interest. One of the samples of linseed oil used in my experiments was aerated alone in the form of an emulsion for long periods. After 12 hours it showed no tendency to autoxidise. After 20 hours however when a sample of the emulsion was transferred to a Barcroft apparatus it displayed straightway without any addition a slow but maintained uptake of oxygen. This was at once greatly accelerated on the addition of a little GSH.

[2] Armstrong and Hilditch [1925] have shown that the simple esters (of oleic acid) are much more resistant to the action of oxidising agents than is the free acid.

are present initially, the oxidising system may not be established. The SH is rapidly oxidised and all uptake may then cease.

The longer the induction period displayed by a sample of oil at p_H 3 to 4, the higher the concentration of SH required to establish an active system at p_H 7·6.

What then is the actual state of affairs during the highly characteristic linear uptake? Is the oil at this stage undergoing autoxidation, the period of induction having been much shortened by the conditions, and obscured by the preliminary oxygen uptake of the SH group? Or, is the efficient promoter of oxidation during the linear period a highly active compound formed by the traces of iron present with that very small residual amount of glutathione which is found to exist in the system during this later stage of the oxygen uptake?

The direct promotion of oxidation by iron cannot be ignored here, because, unlike that of fatty acids, the oxidation of linseed oil is definitely promoted by moderate concentrations of iron in neutral or slightly alkaline systems. It is sure, however, that the minute amounts present in the glutathione preparations used are not by themselves responsible for the phenomena under discussion, since when preparations of the dipeptide are oxidised by aeration they have no effect upon the system when added during any stage of the course of uptake, though the iron content is not altered.

In oil emulsion systems the use of cyanide to inhibit any possible influence of iron offers certain difficulties. The perfectly smooth curves obtained in its absence are apt to become irregular. It is certain, however, that cyanide does not prevent the oxidation of linseed oil under the influence of glutathione preparations at p_H 7·6. When special care has been taken to avoid any change in the p_H as the result of its addition, I have found that even with concentrations of cyanide so high as $M/12$ the effect has been relatively slight. Curves have been obtained precisely similar in form to those seen without cyanide, though usually the velocity of the linear period is somewhat lowered (Fig. 5).

I have obtained no evidence upon which to base any clear explanation of the behaviour of these glyceride systems when neutral or slightly alkaline; but the facts which indicate such marked differences in the behaviour of free fatty acids, or their sodium salts, and their glycerides, respectively, when undergoing oxidation (as emulsions) under the influence of an agent present in living cells, seemed to deserve record. It is of some biological interest to know that lecithin behaves like the former rather than like the latter.

(The data were obtained by the use of three different samples of linseed oil, a sample of pure linolenic acid from Kahlbaum—of which I possessed only a small quantity and could obtain no more—a supply of pure linoleic acid (Kahlbaum) and one of mixed acids, of iodine value 162, carefully prepared from linseed oil[1] and given me by Mr N. K. Adam.)

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[1] It is clear that the observations upon linseed oil described in this section should be repeated upon purified individual glycerides.

V. Oxygen Uptake (with Glutathione) of Lipoid-free Preparations.

As the result of his studies with thioglycollic acid and cysteine, Meyerhof held that the induced oxygen uptake of a thermostable preparation of muscle after thorough extraction with alcohol and ether is so small as to lack significance. I have no doubt that this was mainly due to the hydrogen ion concentration of the systems he studied[1].

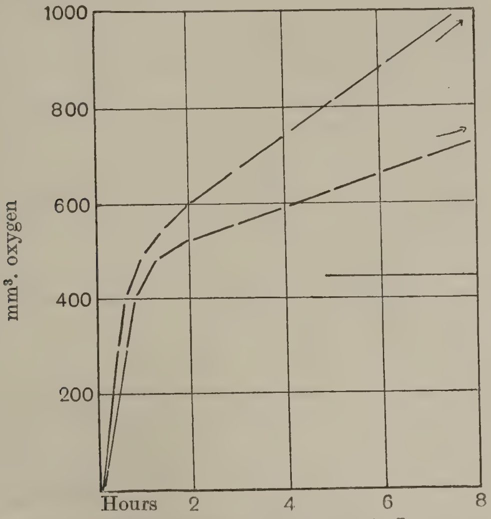

Fig. 5. Uptake of linseed oil with GSH at p_H 7·6 (upper curve). Lower curve the same in presence of *M*/12 KCN. The horizontal line indicates the oxygen equivalent of the SH present. The uptake remained strictly linear till the 15th hour when the observation ceased.

A preparation from washed muscle after very thorough extraction gives in the presence of glutathione at p_H 3·5 no uptake at all. At p_H 7·6, however, it displays an uptake which is never less than one-third of that of an unextracted preparation. It may, indeed, constitute a considerably larger fraction of the latter.

This noteworthy effect of change in p_H shows that the material oxidised in the extracted material is not residual lipoids. It bears also on the nature of the mechanism involved.

Two precautions in technique increase the uptake of such preparations. The first relates to the method of washing the original muscle. If, as recommended by Thunberg in his classical paper on hydrogen donators [1920], the chopped muscle be shaken with water in stoppered cylinders, it is essential that the first washing should be with as large a bulk of fluid as possible and that this should be quickly renewed. So long as the concentration of glutathione in contact with the muscle is appreciable (and most of the substance comes out in the first washings) shaking with air involves an oxidation[2] which appreciably reduces the uptake of a final preparation. Later washings than the first have much less effect. The second point in technique is to avoid oxidation due to peroxide formation in the ether in the residue undergoing extraction. In my own experiments thermostable muscle powders were extracted in a Soxhlet apparatus, first for 8 hours with absolute alcohol; then for a similar period with thoroughly dry ether, and finally for a few hours with alcohol again. The ether is thus removed before the preparation is taken out of the Soxhlet tube. The alcohol is removed in a vacuum desiccator.

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[1] Meyerhof [1923, p. 542] does not mention the p_H of his media in this section of his paper, but it is likely from internal evidence that the observations were made on the acid side of neutrality.

[2] The "fixed SH" group is partly oxidised.

In Fig. 6 will be found typical illustrations of the oxygen uptake at p_H 7·6 of lipoid-free preparations obtained as above. None of them showed any appreciable uptake in the absence of glutathione. It should be observed that all the curves of Fig. 6 were obtained by adding the glutathione in its disulphide form. This means, as will be later more fully demonstrated, that the extracted, lipoid-free tissue retains intact the ability to reduce the disulphide, a circumstance which together with the special influence of the hydrogen ion concentration already mentioned becomes of importance when the nature and mechanism of oxidation in the muscle residue are being considered.

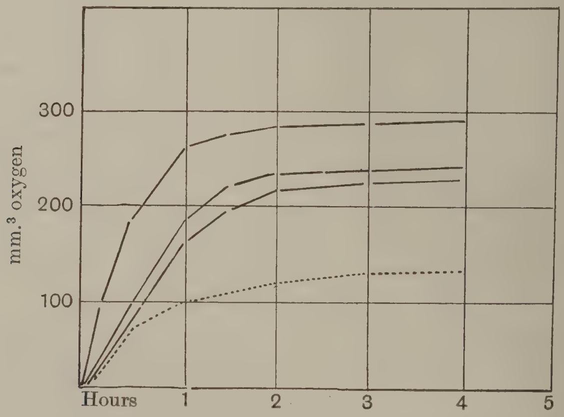

Fig. 6. Uptake of lipoid-free muscle preparations (0·2 g.) at p_H 7·6 with G_2S_2 ($M/200$). The dotted curve was obtained from the preparation which originally yielded the middle curve. It had been kept at room temperature for 3 weeks.

It will be later shown that the oxidation involves the proteins of the tissue. As however most of the observations were made upon muscle before this fact was known, it will be convenient to ignore it for a moment, while some experimental results are discussed in terms of the muscle residue as such.

The oxidation of the fat-free material which is induced by contact with glutathione depends in a remarkable way upon the existence in the former of what was described by Dixon and myself [1922] as the "fixed SH group." It is especially characteristic of muscle as a tissue that after the whole of the soluble glutathione has been removed from it by thorough washing, the insoluble residue still gives a strong nitroprusside reaction. It may perhaps be said that no absolute proof has been given that this reaction is, as a matter of fact, due to an SH group. By a process of exclusion, however, and more particularly by consideration of certain facts dealt with in this paper, the assumption will be found to be justified. According to Abderhalden and Wertheimer [1923] the intensity of the reaction is increased in rigor mortis and diminished by denervation of the muscle during life.

It is essential to any understanding of the oxidations controlled by glutathione, that certain facts concerning the fixed SH group[1] should be fully described. They are curious and necessitate a somewhat detailed discussion.

The fixed SH group is resistant to oxidation by molecular oxygen. Washed muscle or a powdered thermostable residue giving a strong nitroprusside reaction may be suspended in water, or a buffer solution, at p_H 7·6 and aerated for many hours without any appreciable change in the intensity of the reaction. When aerated, however, under conditions otherwise similar but in the presence of glutathione (GSH, or G_2S_2; 50–100 mg. per g. of tissue) the colour reaction progressively diminishes, till after 5 or 6 hours at 37° no reaction is given either by the tissue or the fluid in which it is suspended.

Moreover, the tissue, even when under strictly anaerobic conditions, can with equal ease be deprived of its power to yield the nitroprusside reaction. If it be suspended in an aqueous solution of oxidised glutathione at p_H 7·6 in a fully evacuated vessel the reaction is found progressively to disappear from the tissue, while, under these anaerobic conditions, the solution, which of course gives none at the outset, comes to display an intense reaction.

It is important, however, to make it clear that in this phenomenon equilibrium relations are displayed. The transference of the colour reaction (to use for the moment a non-committal phrase) from tissue to solution may cease while it is incomplete. Before the tissue ceases to give any observable reaction, it must have been exposed to G_2S_2 not only for a sufficient time, but to a solution of sufficient volume and concentration.

The following describes an observation, typical of many, illustrating the above-mentioned facts. 1 g. of thermostable muscle powder giving a strong nitroprusside reaction was placed in a vacuum tube with 20 cc. of $M/100$ G_2S_2. The tube was evacuated and allowed to stand, with occasional shaking, for 3 hours at 37°. On opening the tube the fluid was found to give an intense reaction, whilst a sample of the tissue after thorough washing yielded one which was still definite, though relatively slight. The tissue and fluid were then returned to the vacuum tube which was again exhausted. No further change occurred; the system was already in equilibrium. The original fluid was then replaced by a second quantity (10 cc.) of G_2S_2 solution. After 2 hours the solution gave a slight reaction, whilst the washed tissue now gave no reaction strong enough for observation.

A tissue preparation which, as the result of the treatment described, gives no observable nitroprusside reaction again yields an intense reaction after contact with solutions of thiol compounds in sufficient concentration. This restored colour reaction, like the original, is still displayed with undiminished intensity after the most thorough washing of the tissue with water.

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[1] The term is not a particularly happy one, but it is convenient in discussion. It has proved necessary to use it very frequently in this paper.

Effective in producing the above result are solutions (*circa* $M/10$) of reduced glutathione, cysteine, and thioglycollic acid. As will be later shown, the intensity of the reaction as thus restored increases up to a limit with the concentration of the SH solution to which the tissue has been exposed. There is no doubt that a "fixed" SH group is in this way restored to any tissue preparation which by contact with the disulphide form of glutathione has previously lost this factor. Like that in the original tissue, the restored group is, as already stated, quite irremovable by washing. It is resistant, like the former, to direct oxidation, but disappears in like manner as the result of contact with solutions of G_2S_2. By repeating the treatment just described it may be again restored. A fact of special note is that a muscle preparation which on the loss of its original fixed thiol group ceases to take up oxygen in the presence of glutathione is, after the restoration, capable of a renewed uptake greatly in excess of the oxygen equivalent of the SH restored. This matter is fully dealt with later.

An endeavour has been made to throw further light on the nature of the phenomena just described. On reading the above description, or on observing the phenomena for the first time the impression might be left that the thiol compound used to restore the SH group is merely adsorbed by the tissue residue; or—since the "restored" group in the latter withstands all washing—that it has become fixed at some special surface in the muscle. If this view were justified, it might be further assumed that the original nitroprusside reaction of washed muscle is due to "fixed" glutathione. The distinction between the soluble dipeptide, which can be so readily washed out, and the molecule, or structure, carrying the SH group, which no amount of washing affects, is doubtless sharp. It must be remembered, however, that the muscle fibre is heterogeneous. While the greater part of the glutathione may be simply dissolved in the fibres, a part might be fixed owing to the properties of a special surface. When by contact with solutions of G_2S_2 the nitroprusside reaction disappears from the tissue and reappears in the solution, it might be because the disulphide form, when in sufficiently high concentration, is able to replace the thiol form (or another thiol compound) at the surface in question. Such a view is perhaps unlikely, but I find as a result of discussing the facts with others that it is necessary to consider it. It can easily be disproved, however.

A tissue preparation which has lost its nitroprusside reaction as the result of contact with a solution of G_2S_2 will again show a reaction upon treatment with sulphites. The best result is got by warming it with sodium hydrosulphite. The reaction is never so intense as when it is restored by exposure to solutions of GSH, etc., but it is similarly removed by fresh contact with a G_2S_2 solution which then itself shows a typical reaction. Hydrosulphites induce colour changes in nitroprusside solutions, but give no reaction which could possibly be confused with that of the SH group. When a nitroprusside reaction has been restored to the tissue by exposure to a thiol compound and when the reaction is found to be transferable to a solution of G_2S_2, it is easy to show that this is not because the latter liberates the thiol compound from some previously established association with the tissue. Clear proof can be obtained by the use of thioglycollic acid. This substance is extracted from acid aqueous solution by ether, but can be easily removed from the ether by shaking with a small volume of alkaline water. In the circumstances of the experiments under description, it is therefore easy to decide whether it be or be not present in a G_2S_2 solution. Neither form of glutathione is soluble in ether.

The nitroprusside reaction was restored to a muscle preparation (2·0 g.) by exposure to a thioglycollic acid solution ($M/10$) at p_H 6·8 for 3 hours. After the usual thorough washing, it was suspended anaerobically in 10 cc. of G_2S_2 solution ($M/50$, p_H 7·4) for 3 hours at 37°. The solution, which then showed an intense reaction, was made slightly acid with sulphuric acid and shaken with 10 times its volume of ether. The ether after filtration through three dry filter papers, to remove traces of the aqueous phase, was then shaken with 10 cc. of water made slightly alkaline. The extract, after filtration through two wet filter papers to remove traces of the ether phase, was tested for the nitroprusside reaction. In spite of its extreme delicacy the reaction was entirely absent. At least 8 mg. of thioglycollic acid must have been present, were this substance responsible for the amount of SH in the solution; whereas in a control experiment, when 3 mg. only were added to the same bulk of G_2S_2 solution and the above processes applied, the aqueous extract from the ether gave an intense nitroprusside reaction. This experiment has been many times repeated; always with the same result[1].

It is clear that in the experiments just described the observed transference of the nitroprusside reaction from tissue to solution could not have been due to the replacement of adsorbed thioglycollic acid. Now the general properties of the fixed SH group are the same in the original washed muscle, and in preparations to which it has been restored by contact with solutions of thioglycollic acid, cysteine, or reduced glutathione.

It is sure, therefore, that the phenomena under discussion are not due to interchange between molecules in solution and molecules fixed under reversible conditions in the tissue. The only alternative is to recognise that they involve actual oxidation and reduction. The carrier of the fixed SH group in the tissue must be able to reduce glutathione when, as a disulphide, it is present in solution, and itself undergo oxidation by the latter. On the other hand, the tissue factor when oxidised (presumably to a disulphide form) can upon change of concentrations be reduced by thiol compounds in solution. It must, of course, be remembered that the molecular associations of the reacting sulphur groups are not the same. The result of such reversible relations must be the establishment of equilibria depending upon the relative concentrations of SH and S-S in solution and the activity of similar groups in the solid phase.

With reversible reactions occurring in a heterogeneous system of this kind equilibrium conditions must be complex, and their quantitative study (especially having regard to the nature of the materials) by no means easy. Some estimations dealing with the exchange of the SH group between the solution and the tissue have been made, however.

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[1] I owe to Mr D. C. Harrison the suggestion that the properties of thioglycollic acid would make the above test available. When it is used as above to reduce a tissue preparation, prolonged washing is required for the removal of adsorbed traces.

Oxidised tissue preparations giving no nitroprusside reaction were exposed to solutions of cysteine, reduced glutathione or thioglycollic acid in varying concentrations. By means of titrations with iodine ($N/100$) the diminution in the SH group of the solutions was determined. Since the concentrations were sometimes high and the change relatively small, estimations by difference were not satisfactory. Moreover, when the observations are made at p_H 7·6, the thiol compounds are apt to oxidise during the processes of measurement, etc. The tissue preparations were therefore first reduced by anaerobic exposure to a solution of the thiol compound and after thorough washing were suspended, also under anaerobic conditions, in a solution of the disulphide form of glutathione, sufficient in amount to secure complete oxidation of the SH group in the tissue. As the amount of SH which then appears in the solution is proportionate to, and, as will later be pointed out, probably identical with that previously in the tissue, direct titration of the solution gives the information required. The following are typical observations.

A thermostable muscle powder in quantity was oxidised until it gave no trace of a nitroprusside reaction, and was then washed, dehydrated with alcohol, and re-dried in a desiccator. 1 g. was placed in each of a series of vacuum tubes containing 5 cc. of aqueous solutions of thioglycollic acid of varying concentrations (see below). The acid was neutralised with sodium carbonate (p_H 7·6). After 3 hours in evacuated tubes at 37°, the tissue residues were filtered off and very thoroughly washed, until the washing gave no trace of a nitroprusside reaction. Each lot was then placed in a fresh vacuum vessel and covered with 20 cc. of G_2S_2 solution ($M/50$). The tubes were evacuated and stood with occasional shaking for 3 hours at 37°. They were then opened and their contents immediately made slightly acid with sulphuric acid to stabilise the SH group. The tissue residues were then filtered off and washed, and the filtrates titrated with $N/100$ iodine; starch being used as an indicator. The results were as follows:

Concentration of the thioglycollic acid solutions	$M/100$	$M/50$	$M/20$	$M/15$	$M/10$
cc. of $N/100$ I_2 required	0·95	1·90	3·3	3·7	4·7

Although the titration figures in this experiment are small, they are reliable and consistent with the results of other experiments of the same type. They lie on a smooth curve and when cysteine or glutathione was used to reduce the oxidised tissue similar curves were obtained.

In one experiment G_2S_2 solutions of different concentrations were placed in contact with equal weights of muscle powder (containing its original fixed SH) and, after equilibrium was established anaerobically, the GSH in solution was determined, as before, by iodine titration.

	$M/100$	$M/50$	$M/50$	$M/10$
Volume and concentration of G_2S_2 in contact with 1 g. muscle residue	5 cc.	5 cc.	10 cc.	5 cc.
cc. of $N/100$ I_2 required	2·4	3·1	3·4	4·2

The figures of these experiments are reproduced as they demonstrate clearly the existence of equilibria between glutathione in solution and sulphur groups in the insoluble muscle residue. No more than this is claimed for them.

The important influence of the fixed SH group upon the oxygen uptake of the lipoid-free muscle residue now calls for discussion. There is no uptake without contact with glutathione in solution; there is also no uptake without the existence of the SH group in the tissue itself. Seeing then that the disappearance of the latter is so characteristic an indicator of oxidation by G_2S_2, the suggestion might arise that its oxidation is responsible for the whole oxygen uptake. This however as will be shown immediately is certainly not the case.

On the other hand, it seems likely that the SH group is the only reducing agency left in the thermostable residue, and probably the only factor, even in muscle which has been simply washed, which reduces glutathione. Tunnicliffe [1925] found that the oxygen equivalent of the SH which appears in G_2S_2 solutions after reduction by 1 g. of thermostable muscle powder is of the order of 140–150 mm.^{3}. Such figures I have frequently obtained. When, however, the original muscle has been washed with the precautions mentioned at the beginning of this section, and when (remembering the equilibrium relations) care is taken to see, by obtaining a negative nitroprusside test, that the tissue is completely oxidised[1], the above value comes nearer to 200 mm.^{3}. I do not find that it is altered by removal of the lipoids if care be taken to avoid oxidation during extraction by ether. Now, if one estimates by iodine titration, with such accuracy as is possible, the oxygen equivalent of the SH which disappears from a strong GSH solution ($M/10$, or higher) when employed to restore the SH group to 1 g. of an oxidised preparation, it again proves to be of the same order. That the SH group alone is the essential reducing factor in the thermostable muscle residue, and that the above data give therefore a measure of its influence become nearly certain when the phenomena under discussion are found as will be immediately shown to depend upon the properties of protein.

If the thiol group be the sole reducing factor acting in the system constituted by the muscle residue and glutathione, it is certain that the capacity for oxygen uptake of the system is greatly in excess of the 200 mm.^{3} per g. dry tissue which represents the oxygen equivalent of that group. The former, as we have seen, is of the order of 800–1000 mm.^{3} per g. or even more. Moreover, it is limited to this amount solely because during the oxygenation of the system under the experimental conditions the fixed SH becomes itself oxidised long before other processes of oxidation which it promotes, and for which its presence is necessary, are complete. If a muscle residue, which has been oxidised by G_2S_2 on lines which have been fully discussed, so that it no longer gives a nitroprusside reaction, and, in consequence, has ceased to take up oxygen, be again completely reduced by contact with a strong solution of GSH, etc., and thoroughly washed, it is found to display a fresh uptake which may be of the order of 2000 mm.^{3} (in the case of lipoid-free preparations) per gram. The amount of this uptake depends upon the concentration of the thiol solution to which the preparation has been exposed; that is, upon the extent to which it has been reduced and its fixed SH group restored. The above figure is obtained after contact with GSH in solutions of $M/10$, or upwards. After this second uptake has ceased, fresh reduction again induces a fresh capacity for the absorption of oxygen (always necessitating the presence of glutathione) which may again amount to a figure of the above order! It will now be understood that the actual uptake of a lipoid-free muscle residue as first prepared will depend upon whether its fixed SH group is at a maximum or not.

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[1] Szent-Györgyi [1925] assumes that the fixed SH is not oxidised by G_2S_2. He evidently did not observe that the reaction comes to an equilibrium and in any case takes time to complete.

The following table illustrates the nature of the quantitative data which have been obtained during a study of the phenomena in question. Preparation A was a fat-free thermostable powder, prepared without special precautions and used some weeks after its preparation. In the preparation of B, an otherwise similar powder, care was taken at each step to avoid oxidation, and it was used immediately.

Table III.

Thermostable preparations from rabbit's muscle, extracted with alcohol followed by ether. "A" a stock preparation, "B" a preparation made with careful avoidance of oxidation (see text) and used when fresh.

Treatment of material before determination of uptake	Total O_2 uptake when shaken in G_2S_2 solution ($M/200$) at p_H 7·6; mm.^{3} per g.	
	A	B
Original preparation before removal of lipoids	2100	—
Extracted (alcohol-ether-alcohol)	710	1020
Oxidised aerobically with G_2S_2 till free from nitroprusside reaction	±40	0
Reduced in GSH solution ($M/10$)	1010 +	1850
Oxidised anaerobically with G_2S_2	0	—
Reduced in cysteine solution ($M/10$))	1810	—
Oxidised aerobically and then reduced by thioglycollic acid ($M/10$)	—	2010

V *a*. UPTAKE OF PURE PROTEINS.

It will now be shown that the special characters of the fat-free muscle residue which have just been discussed are due to the properties of its proteins, and that the oxidations which occur in it involve apparently no other materials.

Earlier observations bear upon the matter, but I think the significance of the known facts is much increased in the light of the observations to be described.

Arnold [1914] observed that muscle proteins give a nitroprusside reaction, though at the time it was somewhat of an assumption that this was due to the proteins as such. Especially intense, he found, was the reaction in the case of the least soluble proteins (the "myostromin" of Danilewski). The proteins of blood plasma and those of "excretions and secretions" gave no reaction. Heffter [1907] had previously shown that blood proteins, serum globulin at any rate, can be made to yield a reaction on treatment with sodium sulphite. Gelatin, caseinogen, egg-globulin and fibrin did not display this property.

Heffter showed that egg-albumin, which gives no reaction in its natural state, acquires the power to do so after denaturation. This remarkable accompaniment of denaturation formed the subject of an extensive study by L. J. Harris [1923] in this laboratory. He advanced some suggestive hypotheses in explanation.

Abderhalden and Wertheimer [1923] have made the interesting observation that when egg-albumin is coagulated by heat, and shows in consequence a nitroprusside reaction, it can form (with cystine) a system which takes up an appreciable amount of oxygen (45 mm.^{3} per g. per hour). The total uptake, however, is, I find, very small.

The nitroprusside reaction as conferred upon certain pure proteins when, as coagula, they are brought into contact with strong solutions of thiol compounds is very much more intense than can be produced by denaturation processes alone, even in the case of ovalbumin, which among native proteins offers the only case of a marked reaction developing on simple coagulation by heat. The proteins of blood serum which develop no trace of a reaction on denaturation, acquire one of peculiar intensity when afterwards treated with GSH in high concentrations. The amount of the reactive SH group thus established in the protein complex varies with the nature of the protein as will be immediately shown. It seems to be a definite characteristic of each individual protein.

In every case the group when established has the properties described for it in the case of the muscle proteins. That its appearance is due to a reduction in the protein complex, and not to any form of adsorption of the thiol compounds used to produce it, can be demonstrated on the lines already described. It is highly resistant to oxidation by molecular oxygen in the absence of glutathione or its equivalent, whilst it is oxidised by G_2S_2 (and of course reduces the latter) under the conditions defined above.

The establishment of the SH group in its molecule determines in every susceptible protein a capacity to take up oxygen in amount which, if not high in a chemical sense, is high in relation to the phenomena which are under review. It is indeed not yet sure what are the limits of the oxidation involved.

Experiment has shown that, as already stated, the maximal production of the SH group on reduction varies from protein to protein, but the data obtained have not yet been sufficiently controlled to give them a strictly quantitative value. The matter is under study. To take one example: after treating coagula from (*a*) mixed serum proteins wholly free from lipoids, (*b*) pure crystalline egg-albumin, and (*c*) crude fibrin, with $M/10$ solutions of GSH or thioglycollic acid, I found that the oxygen equivalent of the SH group established in (*a*) (determined as described above) was of the order of 300 mm.^{3} per g. protein, of that in (*b*) 160 mm.^{3}, and of that in (*c*) at most 60 mm.^{3}. It is perhaps not sure that really pure fibrin displays the phenomenon at all.

Gelatin is incapable of developing an SH group. No protein or protein preparation which fails to exhibit a nitroprusside reaction shows any tendency to reduce solutions of G_2S_2.

The oxygen uptake of the reduced proteins in the presence of glutathione at p_H 7·6, while more or less proportionate to the amount of SH established, is, as already stated, greatly in excess of the actual oxygen equivalent of this. The specimen of mixed serum proteins just referred to, prepared free from lipoids by the ice-cold alcohol method of Hardy and Gardiner [1910] was exposed in powdered form to strong solutions of reduced glutathione and thioglycollic acid, respectively ($M/5$ in each case). Each portion was then washed with water till no trace of nitroprusside reaction was given by the washings (whenever thioglycollic acid is used this process is prolonged) and dried in a vacuum desiccator.

The oxygen uptake of each portion in the presence of G_2S_2 was then determined at p_H 7·6 in a Barcroft manometer; the two instruments being shaken side by side. (There was no uptake alone, and none with glutathione at p_H 3·5–4·5.) The uptake at p_H 7·6 was exactly the same in each case, suggesting that the maximal reduction had been produced. The figure obtained was 2350 mm.^{3} per g. protein. In Fig. 7 the curves of uptake in this experiment are given to show how definite is the condition established, and also to show that, when properly standardised, the Barcroft apparatus gives highly consistent results. The two curves are practically superimposable.

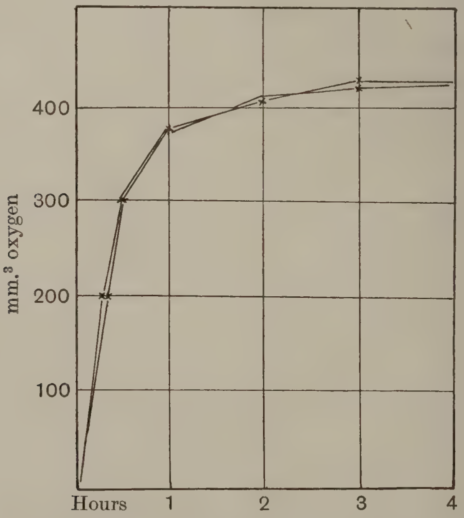

Fig. 7. Uptake of lipoid-free serum proteins (0·2 g.) after coagulation and reduction with GSH and thioglycollic acid; suspended in G_2S_2 $M/200$; p_H 7·6.

But it was easy, as in the case of the lipoid-free muscle residue, to show that the oxidation of the protein stopped at the above figure only because the fixed SH group was exhausted. Freshly reduced by GSH it showed a second uptake of nearly 2000 mm.^{3}. Reduced a third time it showed a further uptake nearly as large, each oxidation involving, of course, the simultaneous influence of glutathione (G_2S_2) in solution. I have not yet determined what is the limit to these successive processes. If each successive uptake corresponded only to the oxygen equivalent of the SH group as originally present, or as re-constituted, the significance of the facts would be less. It is, as a matter of fact, at least ten times as great. If therefore it should prove that the processes of alternate reduction and oxidation can be yet further extended, the final amount of oxidation involved must be great enough to produce an effect upon the protein such as should be easily detected. This matter is being fully investigated[1].

One other aspect of these curious relations must be emphasised. In order that the various proteins should be reduced by contact with thiol solutions they must be in the form of a solid phase. If, *e.g.*, native serum be mixed with a high concentration of GSH and allowed to stand anaerobically, and if after some hours the mixture be diluted, the proteins being then coagulated by heat, filtered off, and quickly washed, the coagulum shows no trace of a nitroprusside reaction. None, moreover, is shown by the precipitate obtained by saturation of the mixture with ammonium sulphate, if it be first thoroughly washed by a saturated solution of the salt. The most probable explanation of this circumstance is that in the process of coagulation a molecular change occurs which is necessary to establish the group which is susceptible of reduction. If this be so, it may appear to reduce the biological significance of the facts. It must be remembered, however, that the proteins which are left in muscle after thorough washing and which constitute an important part of the permanent structure of the fibres, are susceptible of reduction in their native state. If muscle after simple extraction with water be aerated in the presence of G_2S_2 at p_H 7·4 it may be completely oxidised and deprived of its nitroprusside reaction. This is then normally restored by exposure to GSH solutions. It would be interesting to find that the less soluble among tissue proteins share some special character with blood-proteins rendered insoluble by denaturation.

VI. FACTORS CONCERNED IN THE OXIDATIONS DESCRIBED.

In Section III evidence was advanced to show that in the oxidation of fatty acids by glutathione the reaction changed in type with change of hydrogen ion concentration. In acid systems the thiol group acts essentially as an oxygen carrier, in neutral or alkaline systems its own oxidation involves the fatty acids in a coupled or induced reaction. Does the oxidation of protein conform to either of these types, or is it rather a process to be conveniently viewed from the standpoint of Wieland as one of hydrogen activation and transport? Some facts seemed to suggest this, but others offer difficulties. It must indeed be admitted that the ascertained facts are insufficient to make clear the nature of the phenomena.

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[1] Mr A. Carruthers has found that as the result of four successive reductions and oxidations a sample of lipoid-free serum proteins took up 10 cc. per g. The limit was not then reached however.

In discussing the evidence it will be convenient to deal first with systems consisting solely of protein and the oxidised or disulphide form of glutathione. Only proteins such as those of washed muscle which naturally contain an SH group, or denaturated proteins upon which an SH group has been conferred, suffer oxidation in such systems. It will be convenient to speak of these—for lack of a better term—as "reduced" proteins.

For a sustained oxygen uptake by unsaturated fatty acids, the conditions in the system must, as we have seen, be such as secure stability of the SH group (Section III). For the oxidation of protein, on the other hand, the conditions must favour the autoxidation of SH (Section IV). This distinction is fundamental. In each case the hydrogen ion concentration exerts a dominant action in determining these requisite conditions. In the latter case the presence of dissolved iron in minute amounts may be a necessary factor.

As the disulphide form of glutathione is reducible by the "fixed SH" throughout a wide range of hydrogen ion concentration, fat is oxidised in the presence of "reduced" protein at p_H 3·5, even though G_2S_2, and not GSH, is initially supplied to the system. Experiments in proof of this will be described immediately. On the other hand, in systems containing reduced protein and G_2S_2 alone, there is no oxygen uptake at such a grade of acidity. The disulphide in solution is reduced until equilibrium is established with the SH in the solid protein, and the system then becomes inactive, exactly as though it were under anaerobic conditions. When the p_H is changed to 7·4–7·6, the oxygen uptake already described actively proceeds. It has been shown that this uptake depends upon the existence of the fixed SH group, and also that the total uptake is much in excess of the oxygen equivalent of this.

A significant circumstance in the behaviour of a system consisting of reduced protein (or a thermostable muscle residue) and G_2S_2 is that throughout the course of its oxygen uptake at p_H 7·6 the concentration of SH in solution remains very low. If the process be arrested at successive periods during the course of the uptake and a nitroprusside test applied to the contents of the flask of the Barcroft apparatus, the colour reaction in the protein is found to diminish progressively, whilst that in the solution is very faint throughout, even when the oxygen uptake is proceeding at its highest velocity. By the time, however, the nitroprusside reaction has become negative in both, the uptake ceases. The reason for the low concentration of SH in solution is clear. Compared with the rate of the oxidation of GSH at p_H 7·6 the reduction of G_2S_2 is a slow reaction. Conditions in the system adjust therefore to this relation. Oxidation follows reduction and the process is continuous, but because of the velocity relations the intermediate reduction product never reaches more than a very low concentration. The reduction process is much less affected by p_H than is the oxidation [Tunnicliffe, 1925].

Now although the oxidation of protein by glutathione is so entirely inhibited at grades of acidity such as correspond to a p_H of 3·5–4·0, in the region of p_H 6·5–6·8 it proceeds, though at a velocity somewhat less than at 7·6.

In accordance with the relations indicated the SH concentration in solution is higher during the course of the oxygen uptake in the slightly acid system. At such a grade of acidity the rate of reduction is scarcely affected; that of oxidation is considerably slower.

All these facts seemed to justify the view that in the system now under discussion glutathione is an intermediate hydrogen acceptor. It would seem to share in a process of hydrogen transport of the kind pictured by Wieland and Thunberg. On these lines the smallness of its concentration in an active system offers no difficulties. It is indeed, as was indicated above, to be expected. This was the view put forward in previous communications in respect of the oxidation of washed muscle, or its thermostable residue, by glutathione. I had myself always pictured that there were constituents of the washed muscle oxidised on such lines; constituents, namely, which are capable of reducing the disulphide form; an essential for their oxidation on the lines conceived. It would now seem from evidence discussed in the last section, that the one essential reducing factor is the SH group present in the insoluble muscle proteins. When limited to this factor the above view certainly holds. But what is the mechanism of the oxidation in protein which, while certainly depending upon the presence of the fixed SH group, involves an uptake of oxygen ten times as great as that required to oxidise the SH group itself? It seemed during earlier stages of this enquiry as though the behaviour of the SH group in the muscle residue might only be, so to speak, an indicator, and that other agencies in the tissue also possessed reducing power. But when it was realised that the material oxidised was protein, it was further found that no protein reduces G_2S_2 unless it contains an SH group. Moreover, as was stated in the last section, Tunnicliffe's quantitative studies, and data which I have since repeatedly obtained myself, show that when the muscle residue (or a "reduced" protein) is in contact under anaerobic conditions with solutions of G_2S_2, the maximal reduction of the latter corresponds quantitatively with the reducing capacity of the fixed SH group. Nevertheless, aerobically, the oxygen uptake is ten-fold the oxygen equivalent of this. These results seem to preclude the possibility that the whole process of oxidation is one of hydrogen transport. Yet if oxidation, say at unsaturated linkages in the protein molecule, or the oxidation of structures other than the SH group, depends upon a function of SH in solution (akin, say, to that displayed in the oxidation of fatty acids) it is difficult to understand how it actively proceeds when the concentration of soluble SH in the system is so exceedingly low.

Faced with this difficulty it is perhaps legitimate to consider the following possibility, even if it be unlikely. In the process which results in the oxidation of protein by glutathione, the fixed sulphur group might be an additional intermediate hydrogen acceptor. While the SH in the protein reduces G_2S_2 in solution, it might be necessary, in order that the process should continue, that the fixed SH group should itself be again reduced by other factors in the protein which, while ultimately susceptible of oxidation, cannot directly reduce

G_2S_2. Now, the latter reduction may be a much slower process than the former, and may not have been given time to display its effects appreciably in the anaerobic experiments described in the last section. Some of these were prolonged, however, and the suggestion is unlikely. Yet the following facts should be considered here. If washed muscle be thoroughly aerated in the presence of glutathione until it gives no trace of a nitroprusside reaction, it will upon heating in water to 100° again give a reaction which is quite intense, and it will now again reduce G_2S_2 though, quantitatively, much less effectively than when it has itself been reduced by contact with a thiol compound. The proteins behave like egg-albumin. This shows at least that the reconstitution of the fixed SH group may in some sense result from changes occurring within the solid (protein) phase of the system. But that this reappearance of the SH group does not strictly represent the process postulated above (accelerated by the higher temperature) is indicated by the fact that though it occurs immediately at 100°, and more slowly at 60°, it does not seem to occur, even slowly, at temperatures below the coagulation point of the muscle proteins. It involves denaturation, therefore. But, as was made clear at the close of the last section, the proteins of washed muscle do not need to be denaturated in order to show their normal large oxygen uptake. It is difficult therefore to see how the whole process of protein oxidation by glutathione can be entirely one of hydrogen transport. Provisionally at least, it seems we must look to the SH group in solution as the promoter of oxidation, maintained, as it is, in very low concentration, by the slow reducing action of the fixed SH group.

If, however, GSH be supplied to the system initially (instead of G_2S_2) there is no increase in the oxidation of the protein. What happens is noteworthy. If two Barcroft manometers be shaken side by side, each containing muscle powder and glutathione in Ringer's solution, but with GSH in one and G_2S_2 in the other (all concentrations and conditions being otherwise the same) curves of uptake such as those in Fig. 8 are (at p_H 7·6) consistently obtained.

The system containing GSH shows the higher initial velocity, but this must be due in the main to the independent oxidation of SH. For at a point when the excess of oxygen taken up by this system has become nearly (but not quite) equal to that required for the oxidation of all the GSH added, the two curves become parallel for the rest of the period of uptake. During this later period, which may be prolonged, each system contains the same exceedingly low concentration of SH in solution. This means that in both cases the oxidation of the protein is essentially dependent upon the fixed SH group as the agent which reduces glutathione. The reduction of the S-S form is, as we have seen, a slower reaction than the oxidation of the SH form and therefore finally determines the velocity of oxygen uptake.

Having dealt with the oxidation of fats and proteins separately, a few words may now be said concerning the behaviour of the original thermostable residue from muscle as used by Dixon and myself and afterwards by Meyerhof. Its oxygen uptake has received preliminary discussion in Section III. It is chiefly of interest from the fact that it contains both protein and fat, so that

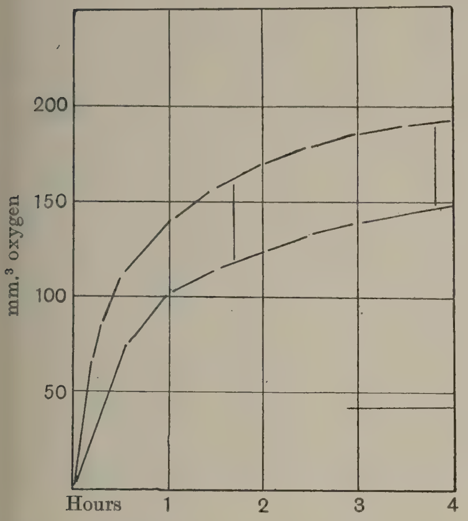

Fig. 8. Uptake of lipoid-free muscle powder at p_H 7·6. Upper curve with GSH, lower curve with G_2S_2. The horizontal line by its position on the scale indicates the oxygen equivalent of the SH initially present in the former system, and the length of the vertical lines between the curves corresponds with this value.

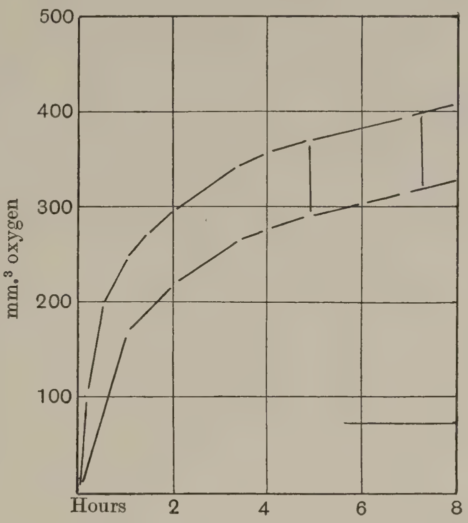

Fig. 9. Uptake of thermostable muscle powder (0·2 g.) at p_H 7·6. Upper curve with GSH, lower curve with G_2S_2. Indications as in Fig 8.

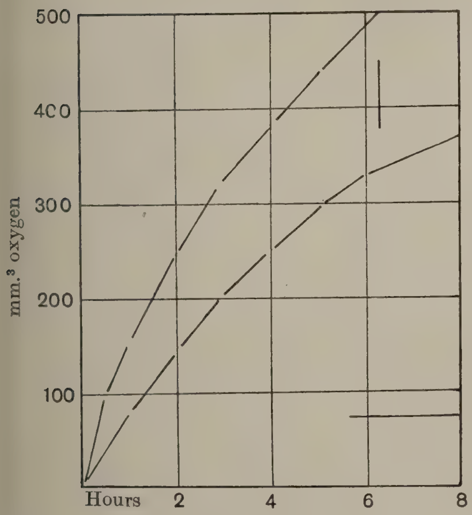

Fig. 10. Thermostable muscle powder (0·2 g.) at p_H 6·0. Upper curve GSH, lower curve G_2S_2. Horizontal and vertical lines show oxygen equivalent of GSH. See Fig. 8.

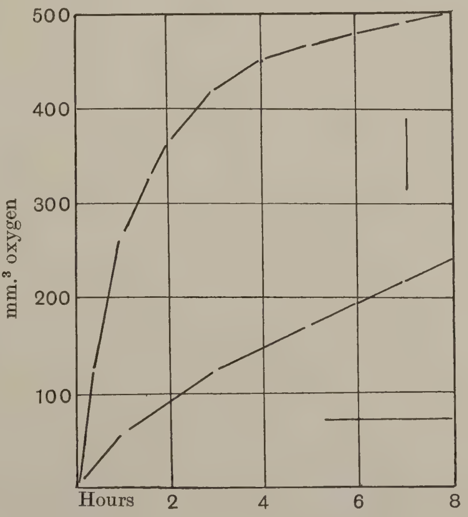

Fig. 11. Thermostable muscle powder (0·2 g.) at p_H 3·8. Upper curve GSH, lower curve G_2S_2. Horizontal and vertical lines show oxygen equivalent of SH.

its uptake varies with changes in hydrogen ion concentration on lines that are instructive. It has been shown in previous sections that at p_H 3·5–4·0 fatty acids are continuously oxidised during the survival of the SH group, while no oxidation occurs in protein. At p_H 7·4–7·6 conditions present secure the peculiar oxidation of protein which has been so fully discussed, while fatty acids undergo only a limited oxidation. With change of p_H therefore different materials predominate in the oxidation and the total uptake may not be the same even with the same preparation. Variation in the fat content of preparations will also have a different influence at different hydrogen ion concentrations. These relations will be sufficiently illustrated by the curves of Figs. 9, 10 and 11, which show the course of oxygen uptake by a muscle powder at p_H 3·8, 6·0 and 7·6 respectively. In each case, the effects of adding GSH and G_2S_2 initially are compared.

The total concentration of glutathione was the same in each system. In one of each pair as compared the dipeptide supplied was partially (but not completely) reduced. The actual oxygen equivalent of the SH present initially is indicated in the figures.

At p_H 7·6 (Fig. 9) the curves are not different in type from those yielded by a lipoid-free preparation (Fig. 8) though the total uptake may be higher. It will be seen that after showing a more rapid initial rise, the curve of uptake with GSH becomes parallel to that with G_2S_2. The oxidation mainly concerns protein, and its velocity in each case is ultimately controlled by the rate of reduction of G_2S_2 by the fixed SH group (see earlier discussion). Hence the remarkably accurate parallelism which is established after the excess of GSH has been oxidised in the system which contained it initially.

At p_H 6·0 (Fig. 10) both fat and protein are oxidised in each system. The curves do not become parallel. In the system which initially contained GSH, an efficient concentration of the SH group is longer maintained and therefore more fat is oxidised than at 7·6. In that supplied with G_2S_2 the uptake depends as before upon the velocity of reduction, but in this more acid medium the SH concentration is again maintained at a higher level, and more fat is oxidised than in the alkaline system.

At p_H 3·8 the curves diverge still more widely. Fat alone is oxidised in either system. In the GSH system the thiol group is stable and fat is more efficiently oxidised than at p_H 6·0. In the G_2S_2 system the oxidation of the fat by SH is controlled by the slow reduction process[1].

These brief references to the curves will be sufficient to indicate the behaviour of systems which contain both "reduced" proteins and fats. The most significant aspect of their behaviour is seen in the influence of protein in determining the oxidation of fat in circumstances which do not secure the oxidation of the latter when it is alone; for instance, when the disulphide is supplied to the system. Without reduction of this there is no oxygen uptake; and fats do not reduce it. But if the system, in addition to the disulphide, contains a muscle residue, or any protein containing the SH group, fat is freely oxidised. This has just been shown in the case of the thermostable muscle residue (Fig. 11, lower curve). The effect is more strikingly demonstrated when excess of fat, in the form of an emulsion, is added to a system consisting of the muscle residue, or a "reduced protein," together with G_2S_2. At p_H 3·0–4·0 a large excess of oxygen is taken up by such a system as the result of adding free linolenic acid, linoleic acid or lecithin, the curve of uptake being linear if the lipoid be in sufficient excess. The velocity, however, is controlled by the relatively slow reduction process. At p_H 7·6 when lecithin or a fatty acid is added to the same system there is an uptake greater than that displayed without them; but the excess is relatively small. This would be expected if the results of Section III are taken into consideration. On the other hand, the addition of glycerides (linseed oil) to the alkaline system results in that linear uptake which results when GSH alone is added to an emulsion. It continues, as in the latter case, with an existing concentration of SH which is exceedingly small (see p. 797). Fig. 12 shows the relation just discussed.

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[1] The experiments yielding these curves have shown how entirely the total oxygen uptake of the thermostable residue (or of water-extracted muscle) depends upon circumstances (see Section III).

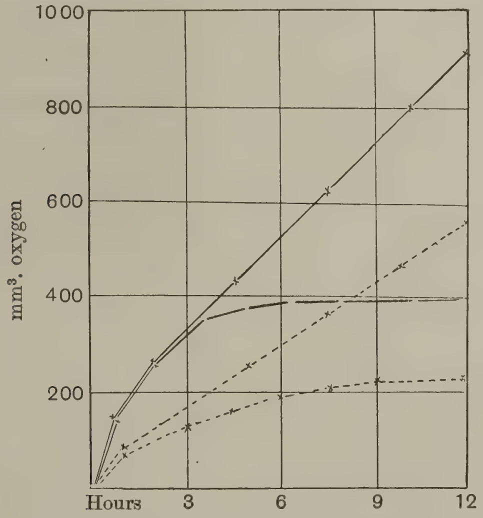

Fig. 12. Curves with continuous line show uptake of a thermostable muscle powder (0·2 g.) plus G_2S_2 with and without the addition of linseed oil; p_H 7·6. Dotted line curves, the uptake of a similar system with and without added lecithin but at p_H 3·5. Each pair of curves was obtained by the use of two Barcroft manometers shaken side by side.

General Discussion.

The experimental studies described in this paper have yielded certain results which were unexpected. Some of the phenomena indeed seem to find no close analogy in the behaviour of chemical systems which are more familiar. It is somewhat surprising, moreover, to find, when a substance presumed to promote biological oxidations in muscle is in question, that its activity should be more easily demonstrable in the case of fats and proteins than in that of carbohydrates or their derivatives. This may be of physiological significance; but it may be rather an accidental result of the particular experimental conditions which have been empirically chosen. The influence of glutathione in promoting oxidations, has, even in cases where its potency has been already demonstrated, shown itself highly sensitive to conditions, and the effect of these has not been by any means fully explored. The highly artificial preparations of the tissue which, in pioneer studies, have been perforce and justifiably employed, are, of course, without many factors which, in the living tissue, may exert a directive influence. One illustration may be mentioned. Miss Thurlow [1925] has shown that the oxidation of the SH group when it occurs in the presence of a peroxidase may induce other oxidations (*e.g.* of nitrites) which do not occur in the absence of this latter factor. The possibilities suggested by this observation are being explored in this laboratory but as the work is being done by others the results are not discussed in this paper.

It will serve the purpose of a brief general discussion of the results obtained in the present research if a comparison be made with those described by Meyerhof in his classical paper on the subject [1923].

Meyerhof was the first to observe the oxidation of lipoids (lecithin, linolenic acid) under the influence of a thiol compound (thioglycollic acid). He was inclined to think that this oxidation might be the only one induced by glutathione in muscle, though in the closing words of the paper just referred to he disavows any finality in that opinion.

He found that the oxidation of the lipoids occurred in acid systems (most effectively at p_{H} 3·0–4·0) and upon the behaviour of such systems he based his view as to the mechanism of the oxidation by SH. He did not observe the oxidation of fats (limited but quite significant) that occurs at hydrogen ion concentrations nearer to that of living tissues (Section IV). Nor did he observe the oxidation of protein which has been described in this paper. In an earlier publication [1918] he was inclined, it is true, to attribute to the oxidation of protein part of the uptake of oxygen displayed by bouillon to which thioglycollic acid had been added. In his later work [1923], however, he rejected this view on finding that serum proteins when thoroughly freed from lipoids showed no uptake. This observation was undoubtedly correct. The native blood proteins show no uptake whatever under the influence of glutathione; but this, as will be understood from facts advanced in previous sections, is because they lack what has been termed throughout this paper "a fixed SH" group. It is of interest indeed to note that the proteins of the fluids of the body, and the albuminoids of connective tissues generally, are immune from the type of oxidation suffered by the proteins of such an active tissue as muscle. They are not exposed to the influence of glutathione, which appears to be absent from the plasma, nor are they exposed to influences which establish in them the second factor necessary for the oxidation in question, namely, the fixed SH group.

But Meyerhof also found that muscle preparations when deprived of all lipoids showed no more than a slight and non-significant oxygen uptake with thioglycollic acid or cysteine. He could not therefore attribute an oxidation to muscle proteins, whereas, as has been shown in previous sections, their oxidation by glutathione is a definite and quantitatively important phenomenon.

The difference between Meyerhof's results and my own is not difficult to explain, however. In the first place the proteins are not oxidised at all in acid systems. Now although it happens that in reporting the results obtained with alcohol-ether extracted preparations [1923, Table IX, p. 542] he does not specify the p_H employed, it is probable, from what comes earlier in his paper, that the systems studied by him were acid. If so, his negative results are fully explained. There is probably a further reason for them, however. If the facts have been made clear in the discussion of Sections V*a* and VI it will be understood that the oxidation of proteins by glutathione unlike that of fats in acid systems, depends upon a succession of oxidation and reduction. That the SH group in solution should be progressively oxidised is essential; but when in any system containing a fat-free muscle residue (or any "reduced" protein) is supplied with SH in excess of a certain low concentration, the excess is (at p_H 7·6) rapidly and (with respect to the protein) ineffectively oxidised. Only when the oxidation adjusts to a slower reduction process; only that is when the properties of the fixed SH group control the velocity of change (Section VI, p. 809) is the effective mechanism for protein oxidation established. Now I have found that neither thioglycollic acid (iron-free) nor cysteine is so efficient as glutathione in establishing such a system. Those who are familiar with Meyerhof's paper will remember that his own experimental studies finally led him to attach a predominant importance to the function of the SH group as an oxygen carrier (being especially active therefore in systems where its concentration is maintained) rather than to the circumstance that it is a reducing agent easily oxidisable to an S-S group, from which it is again easily reproduced by reduction. It was these properties which determined my own previously published views as to the importance of glutathione as a carrier of hydrogen. Nothing, I think, is now more sure than that both views may be correct. With change of conditions glutathione from being an oxygen carrier (oxidation of fatty acids in acid systems) may become, to use a term which is at least convenient, a carrier of hydrogen (oxidation of proteins in neutral or slightly alkaline systems). It is true that, in protein, only the fixed SH group has so far been proven to be a direct source of this transportable hydrogen. The large uptake of oxygen (many times the oxygen equivalent of the above group) which a "reduced" protein displays occurs on lines which are not yet clear. It is at any rate certain that it proceeds only for so long as the fixed SH group survives. Moreover, in any case the influence of this simple reducing factor by itself secures exactly the result which has been pictured [Hopkins, 1923] as following from the ability of tissue agencies to reduce glutathione in solution. It links one form of oxidation to another; *e.g.* that of proteins to that of fats. It is an essential controlling agency. In neutral or alkaline systems the thiol form of glutathione is so rapidly oxidised that, in the absence of a reducing factor, its disappearance quickly renders the system inert. The slow reducing action, for which the fixed SH group is responsible, maintains a concentration of SH in solution, and determines the whole velocity of oxidation. The process continues so long as (and only so long as) the SH in the protein survives. In acid systems its reducing power remains, and, by again securing a continued concentration of SH, determines a prolongation of the oxidising capacity of the system (Section VI). In the cell reduced proteins always co-exist with glutathione. Their coexistence and their mutual influence may well prove to be a biological essential. It must only be remembered that experimental studies have so far been confined to a preliminary analysis of oxidising mechanisms. We have no knowledge of their complex relations in the tissues. In a tissue, for instance, at least after it has been removed from the body and prepared for examination (by which time it has, of course, been in an essentially anaerobic condition for an appreciable interval), the whole of the glutathione is in the reduced form [Tunnicliffe, 1925]. I have indeed found that a tissue in such circumstances—the liver at any rate—will rapidly reduce considerable quantities of additional glutathione when it is added in the disulphide form.

I am well aware indeed that on first acquaintance the curious and at many points obscure phenomena described in this paper may seem to lack biological reality. The experimental results described, however, are constant and easily reproducible. They are yielded by systems which contain actual cell constituents in contact with one another, and depend upon actual properties of these constituents. Such studies would seem to be a necessary, if remote, antecedent to a fuller understanding of the behaviour of the same constituents in the organised phenomena of the living cell itself.

SUMMARY.

In acid systems (p_H 3·0–4·5) glutathione (GSH) promotes the oxidation of certain unsaturated fatty acids and of lecithin. The mechanism of oxidation is, in this case, probably that postulated by Meyerhof [1923].

In neutral or alkaline systems (p_H 7·4–7·6) the nature of the process is different. During the oxidation of the SH group fatty acids are simultaneously oxidised in such a way that equipartition of oxygen occurs.

The oxidation of the glycerides of the above fatty acids proceeds under the influence of GSH, but in detail the process displays remarkable differences from the course of oxidation in the case of the free acids.

Glutathione (GSH or G_2S_2) promotes an oxidation of certain pure proteins on peculiar lines. The oxidation proceeds only when and while the protein itself displays (as shown by the nitroprusside reaction) an SH group. The

proteins of water-extracted muscle contain this group, and suffer the oxidation in question in neutral or faintly alkaline solutions. In acid systems (p_H 3·0–4·5) no oxidation of protein occurs.

In this oxidation the protein SH is oxidised and the nitroprusside reaction disappears, while the total oxygen taken up amounts to ten times the oxygen equivalent of the SH. The protein can then be again reduced; the SH group reappears and (with glutathione) a further uptake of oxygen becomes possible, which is again greatly in excess of the oxygen equivalent of the SH. Successive reductions and oxidations of this kind can be carried out, till finally the protein takes up at least 10 cc. per g. and possibly much more.

At any stage the "oxidised" protein (showing no nitroprusside reaction) can be reduced by contact (as a solid phase) with strong solutions of glutathione (GSH), cysteine, or thioglycollic acid, so that it again displays an intense nitroprusside reaction. Such a "reduced" protein then again takes up oxygen on the lines already described. When insoluble protein is in contact with solutions of glutathione, or other thiol compounds, equilibria are established between sulphur groupings in the solid (protein) phase and sulphur groups in solution. Mutual oxidation and reduction occur as reversible processes.

Whilst the proteins of water-extracted muscle undergo oxidation and reduction, on the lines described, in their native state, certain other proteins (those of blood serum, for example) need to be first denaturated. In an insoluble form the proteins of serum can be so reduced by contact with strong solutions of thiol compounds as to display an intense nitroprusside reaction. They then behave like the muscle proteins.

I owe much to the experimental skill of my assistant, E. J. Morgan. His help is provided by the Medical Research Council and to that body my grateful thanks are due.

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CXXII. ANTINEURITIC YEAST CONCENTRATES. I.

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THE present communication[1] deals with active curative preparations from yeast which have been obtained by the use of polyneuritic pigeons as test animals. The outstanding problems in connection with vitamin B are firstly, concentration of the vitamin, and secondly, whether the properties ascribed to vitamin B reside in one or more constituents. In order to avoid begging the question the word "torulin" is used throughout [Edie, Evans, Moore, Simpson and Webster, 1912] to mean the principle in yeast which cures symptoms of head retraction in pigeons induced by feeding upon polished rice.

TECHNIQUE.

The technique described previously has been used [Peters, 1924]. Test samples have been introduced into the crop. The pigeons have not been fed artificially. Only birds conforming to the conditions previously laid down have been used for the tests. Particular care has been taken to exclude cases of the illusory heat cures, to which allusion was made before. Since then much evidence has accumulated. Birds which happen to get convulsions in the outside aviary must be left for a clear 3 hours before treatment after bringing into the warmer laboratory air. In many cases such birds clear up completely, and do not show symptoms again for a period of 1–3 days. Three days seems to be the limit for these heat cures. We have no explanation to offer for this interesting fact which has been investigated by Roche [1925]. Excluding cases of heat cure there are still found birds which do not fall into the ordinary categories, so that for tests upon important material, it is essential to use more than one bird for standardisation.

Measurement of activity.

We have standardised our results by the use of a curative and protective test, by means of the following equation,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

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[1] A preliminary account of this work appeared in *J. Soc. Chem. Ind.* (1925), 531.

Dry weight here means the weight obtained by drying at 100–105° for 1 hour, after subtraction of the weight of ash. Thus if 4 mg. of a preparation cure and protect for 4 days, the activity is considered to be 100 T.A. Use of this equation implies at least two assumptions: (1) that there are not wide variations in the amount of torulin required by different pigeons, and (2) that no antitorulin factors are present. With regard to (1) it is likely that account must be taken ultimately of both weight and temperature to which pigeons have been exposed. The average weight of the pigeons used by us at the time of convulsions is 270 ± 40 g. Young birds have not been employed. (2) is at present a pure assumption. In practice we have found that the results obtained for times of cure between 2 and 10 days are reasonably quantitative, so much so that it has been possible to follow the distribution of torulin between various fractions with ease. After 10 days the results are not reliable. An example of what is meant is given in Table I.

Table I.

Pigeon	cc. given	Cure and protection (days)
1	0·2	10
2	0·2	9 (after treatment with nitrite)
3	0·2	10
4	0·5	19

Including the nitrite-treated preparation bird 2, 0·2 cc. of this preparation gave in days 10, 10, 9. Upon this basis 0·5 cc. should protect for 25 days, whereas protection only lasted for 19 days.

Table II deals with tests upon an active preparation. It shows what accuracy may be expected from such quantitative tests, and also certain limitations in its use.

Table II.

Pigeon	Dose (cc.)	Cure and protection (days)	Day dose (mg.)
5	0·1	2·5	0·068
6	0·1	2·0	0·085
	0·2	3·0	0·11
7	0·2	4·0	0·085
8	0·3	6·0	0·085
9	0·3	7·5	0·068*
10	0·3	13·0	0·025
11	0·24	2·8	0·24

* This preparation was dried at 100° before administration and found to weigh 0·51 mg.
The average for pigeons 5–9 is 0·084 mg. per day, 1190 T.A.

It will be seen that there are three groups: (*a*) the majority of birds 5–9, which behave well to a standard, (*b*) bird 10 which required much less than the standard, and (*c*) bird 11 which required much more. Bird 10 has been omitted, and represents an abnormality in response, which appears occasionally. It is possible that such birds still have sufficient torulin left in their system, and that the small dose given is enough to set free this store. Bird 11 has also been omitted, for the reason that it was not in good condition when the dose was given, and was also a bird which had been subjected to the tests over a period of 5 months in the laboratory. It must be admitted that at present it is not possible to eliminate the element of judgment in the selection of suitable cases. Use of the equation is however justified by the fact that by its aid we have obtained torulin concentrates some 200 times as concentrated as in the charcoal concentrates. During the progress of the research we have been increasingly impressed by the quantitative response of the majority of test birds to the fractions given.

EXPERIMENTAL.

It is not proposed to give at length the numerous curative experiments made in the course of fractionation. In each case, before proceeding to a further step, results have been checked by the use of birds. A few general statements may be made as to procedures which have not been helpful, when applied to the charcoal concentrates. With phosphotungstic acid it was not found possible by a varied extraction to recover the active principle in reasonable amount. At this stage also adsorption upon kaolin, or upon specimens of fuller's earth procurable by us proved valueless, as did also precipitation with lead acetate in alkaline solution. In the latter case all the torulin present was recovered from the filtrate. We have not been successful in preparing from these concentrates active picrates similar to those described by Seidell [1924].

The following method we have found successful, when working upon a scale of some 7–21 lb., using D.C.L. baker's yeast.

Up to the stage involving the use of acid alcohol to extract torulin from the charcoal, the technique has been the same as previously described [Peters, 1924], with the exception that in later preparations the treatment with BaS has been omitted. Instead charcoal adsorption has been applied direct to the acid fluid after removal of the mercury precipitate. After removal of the first amount of charcoal, the fluid has been neutralised to methyl red and again treated with charcoal in similar amount. The charcoal residues are combined after washing with distilled water, and concentrated. Further treatment can be then carried out as described below. Unless otherwise stated, all precipitates have been removed by the centrifuge and washed with appropriate solution. Concentration has been carried out *in vacuo* under the water pump with a temperature not exceeding 60° in the apparatus described [Peters, 1924].

Stage 1. The charcoal concentrate was diluted with a convenient amount of water and alcohol added to a concentration of 60 %, the precipitate removed and discarded, and the centrifugate (cf.) concentrated to remove alcohol.

Stage 2. The concentrate from stage 1 was diluted with water and 25 % lead acetate added until precipitation was complete, the precipitate removed, the centrifugate and washings combined, treated with HCl to remove excess of lead and concentrated. Any precipitate appearing during concentration can be discarded.

Stage 3. The acid concentrate from stage 2 was treated with methyl alcohol to a concentration of at least 90 % (more does not matter), the precipitate removed, and the centrifugate concentrated to remove methyl alcohol.

Stage 4. The solution from stage 3 was made up to a convenient volume with water and treated with "dialysed iron" 10 %, about 7 cc. being required for a 7 lb. sample. The precipitate was removed by the centrifuge, and washed. This washing was found to be important. Normal NaOH, about 10 cc., was then added until a precipitate ceased to come down, at which stage the fluid is very alkaline. Care must be taken to carry this stage through quickly, and not to allow the solutions to rise above room temperature. We have found in confirmation of the work of others that torulin is inactivated by heating with NaOH. The heavy precipitate is removed and washed with dilute caustic soda. The centrifugate was made acid to litmus as soon as possible and concentrated.

Stage 5. Ethyl alcohol was then added to a concentration of 85 %, the precipitate removed and washed with 85 % alcohol, and the centrifugate concentrated. Alcohol was then added to 95 % and the process repeated. It is best to re-dissolve the gummy precipitates obtained in a minimum amount of water and throw out again with alcohol at this stage, at which there is liable to be loss.

Stage 6. The 90 % alcoholic solution was treated with half a volume of ether, the precipitate removed, taken up in a minimum of water, made up to 90 % alcohol with absolute alcohol, and re-precipitated with ether. The combined centrifugate from the two precipitations was concentrated as far as possible, the extract dissolved in a minimum of water, and made up to a strength of 95 % alcohol with absolute alcohol, any precipitate appearing at this stage being removed.

Stage 7. The fraction obtained was then gradually worked up to higher concentrations of alcohol, the object being to obtain the fraction soluble practically in absolute alcohol. When the fraction soluble in about 99 % alcohol is obtained it is well to take it to dryness, and then treat with absolute alcohol, and allow to stand out of contact with air overnight. About 40 cc. of absolute alcohol were used at this stage for a 7 lb. preparation. The precipitate can then be re-extracted with fresh alcohol. The torulin will be found in the absolute alcohol solution. The final stages require care, and it is well to check the absence of torulin from the final precipitates. The material at this stage is apt to vary. In the case of four preparations which have been worked up to this stage, the activity of the final product has varied between 50–100 T.A. A yield of some 2800 day doses may be expected from 21 lb. of yeast.

Owing to the variability of material, we do not claim that the method described is more than empirical. We have found in working upon the larger scale that it is necessary to repeat stages in order to get these final fractions soluble in alcohol and free from gum. The advantage of separation from solvents lies in the freedom from ash and gum of the final fraction, and the ease with which a false step may be retrieved.

Further treatment of the concentrates.

By fractionation of a product of 63 T.A. in mixtures of ether and alcohol in which the amount of water was reduced to a minimum three fractions were obtained: (*a*) a gummy fraction thrown out of the alcohol by the addition of mere traces of ether, (*b*) an intermediate fraction thrown out by ether up to four volumes of ether to one of alcohol, and (*c*) a fraction soluble in ether-alcohol. The latter contained an oil, comparatively insoluble in water. Fractions (*a*) and (*c*) were relatively inactive. The intermediate fraction when treated with silver sulphate in acid solution threw down an inactive precipitate. From the centrifugate after treatment with silver nitrite in the presence of HCl, ammoniacal silver hydroxide threw down a precipitate, which was extracted with HCl and alcohol, and yielded the preparation of 1190 T.A., tests upon which are described in Table II. This contained ammonium chloride as an impurity, and some evidence has already been obtained that the activity may be pushed to 3000 T.A. This being so, it does not seem unlikely that the activity of torulin itself may prove to lie between 10,000 and 100,000 T.A. or in the region of 0·001 mg. per day for the normal pigeon. This would only bring it into line with substances like pituitrin.

Weight maintenance activity.

It is difficult to know how to relate our preparations to those described by Seidell [1921, 1924] and Emmett and Peacock [1925], both of whom have employed weight maintenance tests. The latter observers describe a preparation, from a source unmentioned, capable of maintaining the weight of pigeons fed upon polished rice in doses of 0·1 mg. per day and of causing gain of weight in doses of 0·15 mg. per day. For the sake of some comparison with the weight maintenance tests, we have carried out the following experiments 1 and 2, of which the details are recorded in Figs. 1 and 2. Exp. 1 was designed to constitute a severe test for protection against polyneuritis, and Exp. 2 to place the bird under a condition in which it should easily show a gain in weight.

Exp. 1 (Fig. 1). Two pigeons upon a polished rice diet were kept outside in the winter weather. For the first 16 days the diet was polished rice alone; following this each bird received every second day 0·17 mg. of the preparation used in Table II. This should constitute a border-line amount to protect against polyneuritis at laboratory temperature. On the 32nd day, as one day was accidentally missed, four doses were given. The weight of the birds steadily fell. Bird 12 died of general weakness on the 44th day without showing symptoms of head retraction. It was found to have undigested rice in its crop. Under the conditions of the experiment the birds might be expected to get symptoms by the 30th day at latest, so that this bird may be considered to have been protected for at least 14 days. Bird 13 lasted until the 50th day, when it developed symptoms of head retraction thereby showing that protection had been afforded previously by the preparation, but that the amount given was rather low. An extra dose of the preparation on this day cured the symptoms, but the bird died of general weakness on the next day, and was found to have no food in the crop. Doubtless not much stress can be laid upon evidence drawn from prolonged feeding upon polished rice at a stage when deficiencies other than the one under study may be present, but it seems reasonable to conclude that protection against polyneuritis may be afforded for many days under severe conditions without weight maintenance.

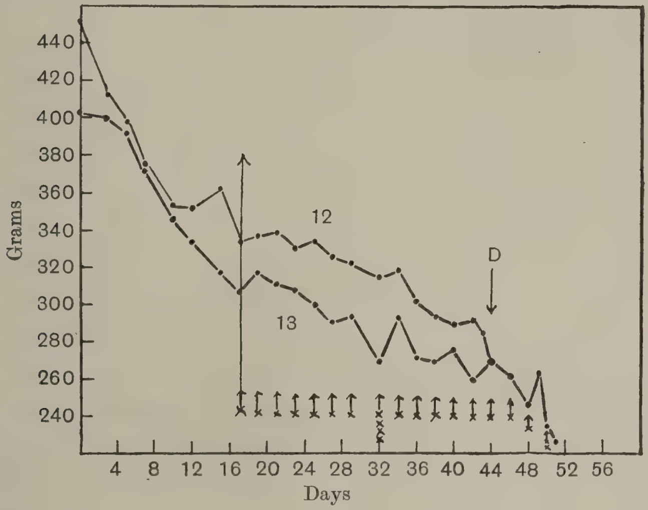

Fig. 1. Exp. 1. Pigeons 12, 13. Ordinates g. weight. Abscissae days. D = death of pigeon 12. × = 2-day dose ≡ 0·17 mg. T.A. 1190.

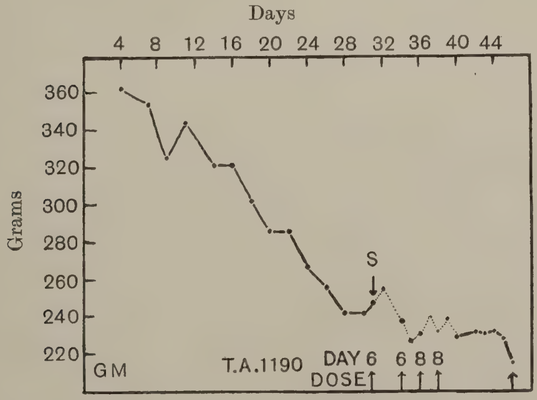

Fig. 2. Exp. 2. Pigeon 14. S = symptoms of head retraction. Diet, polished rice.

Exp. 2 (Fig. 2). During the hot summer weather, bird 14 was kept indoors until it showed symptoms of head retraction on polished rice. After this it was given several doses of the same preparation as in Exp. 1. The time at which symptoms appear in these cases is usually later than under the conditions of Exp. 1. In this case it was the 29th day. The pigeon did not maintain weight even with 3–4 times the day dose, when conditions as regards the general rate of metabolism must have been particularly favourable.

It is clearly necessary to carry out extended tests with other preparations of this nature, but we feel that it is justifiable to conclude that highly active antineuritic preparations can be obtained which have no effect upon the weight curves in doses above the amount necessary to cure symptoms of head retraction. The evidence in this communication therefore supports the view that vitamin B consists of two factors.

SUMMARY.

1. Heat cures must be avoided in working with pigeons suffering from symptoms of head retraction induced by feeding upon polished rice.

2. Using the word "torulin" to mean the principle in yeast curative of these symptoms, the following equation gives quantitative results in most cases, namely,

$$\text{Torulin activity (T.A.)} = \frac{\text{Number of days' protection after cure}}{\text{Weight in mg. of the dry preparation}} \times 100.$$

3. A yeast concentrate has been obtained of 1190 T.A. which cures and protects in doses of 0·084 mg. per day.

4. Doses of this concentrate which protected against polyneuritis for many days did not cause increase in weight of pigeons after cure of the polyneuritic symptoms.

We wish to record our thanks to the Medical Research Council for a grant to one of us (H. W. K.), and for a grant for material for research. We are also grateful to H. Poulter for help with the yeast preparations, and to Clapton for help with the animals.

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CXXIII. RESEARCHES ON THE FERMENTATION OF DRIED TOBACCO.

I. THE METHODS FOR SEPARATING NICOTINE AND AMMONIA.

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(*Received August 26th, 1925.*)

Our object in this research was to separate the nicotine from the other volatile nitrogen-containing bases contained in extracts or expressed juices from tobacco leaves, and to estimate them. In the earlier processes insufficient consideration has been given to this separation.

Kissling's method [1920] as usually carried out has the disadvantage that when extracting the leaves with ether, ammonia among other things also passes into the solvent; comparative tests made by us have shown this to be the case in a manner which places the result beyond the possibility of doubt; further, the distillate obtained by Kissling's method always gives the familiar Nessler's ammonia reaction, whilst pure nicotine gives with Nessler's reagent only a white precipitate, which on the addition of the slightest amount of ammonia becomes coloured.

Toth [Kissling, 1920, p. 98] seeks to avoid the presence of ammonia by allowing the latter to be adsorbed by gypsum, but according to Rasmussen's [Kissling, 1920, p. 99] and Schröder's [1911] investigations the Toth process gives inexact results. König extracts tobacco leaves with toluene and estimates the nicotine polarimetrically. Toth [1911], however, holds this method to be unsuitable because dark-coloured solutions are unpolarisable.

Bertrand and Javilliers [1909] precipitate the nicotine as silicotungstate, decompose this compound in a current of steam and ascertain its amount by titration. This method was unsuitable for our purposes because it did not permit of the estimation of the ammonia; moreover silicotungstic acid is an expensive reagent and difficult to obtain.

Harrison and Self [1912] estimate the ammonia and nicotine obtained by steam-distillation of tobacco after the addition of alkali, absorbing the distillate in titrated acid and then titrating back; they then precipitate the nicotine with iodine and, after destroying the colour of the iodine by means of thiosulphate, distil off the ammonia. The difference between the two titration values gives the nicotine content.

We used the following method. The nicotine, in the mixture of ammonia and nicotine obtained from tobacco leaves by direct steam-distillation, is, after making the liquid weakly acid with sulphuric acid, precipitated by means of a solution of mercuric iodide in potassium iodide (3 g. per 100 cc. water). Under these conditions, ammonia gives no precipitate. The precipitate is allowed to remain overnight and is washed by decantation; it has a wax-like crystalline character. The filtrate obtained is quite clear and free from nicotine. The precipitate can be estimated by the Kjeldahl method, whilst the ammonia can be distilled over from the filtrate in the usual way, an excess of potassium iodide being added before making alkaline to prevent the formation of a Nessler precipitate and consequent loss of ammonia.

For this series of researches it is sufficient to know the total titration value of the volatile nicotine and ammonia, and then, after precipitating the nicotine, to estimate the ammonia content of the filtrate. The difference between the two titration values gives the nicotine content.

The following figures show that this method gives satisfactory results.

We examined a control solution made up as follows:

20 cc. nicotine (pur. prep. Merck) + 20 cc. $N/10$ NH_3 + 60 cc. $N/10$ H_2SO_4.

The nicotine solution was of about 2 % concentration, 10 cc. corresponding to 13·2 cc. $N/10$ H_2SO_4.

(Here and in the following the titrations were made against sodium alizarinsulphonate as indicator.)

20 cc. of this mixture were treated in the way described above.

After distillation the filtrate gave a titre corresponding to 4·4 cc. $N/10$ H_2SO_4. The nicotine precipitate gave after a Kjeldahl determination a titre equivalent to 5 cc. $N/10$ H_2SO_4.

It was found that when one commenced with the same quantity of nicotine, without however adding ammonia, a titration value corresponding to 0·3 cc. $N/10$ H_2SO_4 was always obtained. (Each determination was repeated several times.)

After correcting the experimental value the following data emerge (cc. $N/10$ H_2SO_4):

Value	Nicotine	Ammonia
Calculated	5·3	4·0
Found experimentally	5·0	4·4
Corrected	5·3	4·1

It appears that the nicotine preparation (Merck) contains, though in very small amount, some nitrogen-containing impurities. Thus, for example, pyridine in weakly acid solution gives just the same reaction as nicotine towards KI-mercuric iodide but in contrast to it is readily soluble in excess of the precipitating agent and is therefore driven over and estimated with the ammonia.

The method reduces itself to two distillation processes:

(1) Driving over the nicotine together with the ammonia by steam-distillation.

(2) Distilling over the ammonia out of the filtrate from the nicotine mercury compound (as in the usual NH_3-distillation method).

In the application of this method to tobacco extracts there is of course the danger that the value in the first distillation may be too high, because besides nicotine and ammonia, other more or less volatile bases may pass over at the same time.

If these are also precipitated by the nicotine-precipitating agent, too high a nicotine value will be obtained, since the ammonia values (in the second distillation) will be correct.

If, however, no precipitation occurs, then conversely accurate nicotine values but too high ammonia figures are obtained (*e.g.* with pyridine).

Usually the latter will be the case, so that therefore the value, which when working with tobacco is calculated as NH_3, will generally be more correctly expressed as the value obtained for volatile non-nicotine bases.

Finally, it is to be observed that it is part of our object to find purely physical methods of separation, of practical feasibility, which will next be dealt with.

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CXXIV. RESEARCHES ON THE FERMENTATION OF DRIED TOBACCO.

II. THE ENZYMIC PRODUCTION OF VOLATILE PRODUCTS FROM NICOTINE UNDER THE INFLUENCE OF TOBACCO-LEAF EXTRACTS.

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(*Received August 26th, 1925.*)

INTRODUCTION.

THE decomposition of nicotine during the so-called fermentation process which takes place in the tobacco leaf is one of the most important reactions concerned in this complicated process, as a result of which the raw or dried tobacco becomes converted, in the fermentation stores, into the valuable product of commerce. According to the statements in the literature, decomposition of nicotine also occurs in green tobacco before drying. It is our intention to study the processes taking place during the fermentation, especially to find out the enzymic constituents of the cells of the tobacco leaf which take part in the fermentation. In this connection the literature is lacking in systematic investigations, although individual biochemists express the supposition that oxidases play a part in the process [Loew, 1899, 1900, 1901; Jensen, 1908]. On the other hand, many investigators to-day suggest that the process of fermentation here concerned depends upon bacterial action [Schmidt, 1924; Behrens, 1896, 1901; König, 1900; Suchsland, 1892].

The question is still open and has to be investigated whether or not bacteria, either directly or indirectly, limit the actual fermentation process.

We prepared, as will be shown below, expressed juices and macerates from tobacco leaves under the usual conditions of sterility and examined their effect on nicotine.

We found as a result that definite effects take place, and that volatile nitrogen-containing bases are produced, which can be estimated after distillation.

From their effect on silk peptone (increase of amino-nitrogen) it was ascertained that these juices are able to produce a peptolytic effect. This observation is obviously in agreement with the fact, known in the literature, that protein disappears during the fermentation process. The following researches demonstrate these results.

The problem as to what products are formed from the nicotine on its decomposition is important. In a preliminary investigation we attempted to find whether, after allowing the nicotine to be acted upon for 2 hours at 37° by tobacco extracts, demonstrable quantities of acid are produced.

It was found that the nicotine solution used as substrate had after approximate neutralisation a p_H value of 4·79. The expressed juices, also approximately neutralised, had a p_H value of 5·74. This gives by calculation for a mixture of equal volumes a p_H of 5·05, whereas the electrometric determination gave for this value the figure 5·29. Hence the mixture has become slightly alkaline during the process. Therefore there can be no question of the production of any considerable amount of acid. On the other hand, a preliminary qualitative test showed that we have to deal with the production of carbon dioxide. We also found that the solutions obtained in the decomposition of the nicotine had a remarkably strong odour of pyridine and methylamine. With $KI—HgI_2$ reagent we obtained the familiar white precipitate which is given by solutions of pyridine and which is soluble in excess of the precipitating solution (see previous paper). In all probability the nicotine here undergoes a decomposition process with the formation of pyridine, methylamine and carbon dioxide as end products.

Hence the process must be interpreted as the effect of an enzyme, of the class of oxidases, on the nicotine.

In how far this is to be ascribed to the action of a specific oxidase will be shown by later experiments.

Since the solutions, kept in the thermostat for 2 hours at 37°, showed no change in appearance under the conditions we employed, there can be no possibility of bacterial action.

EXPERIMENTAL.

In the quantitative determination of the decomposition products of nicotine, produced by the enzyme action of macerates and expressed juices of dried and fermented tobacco leaves, the volatile non-nicotine nitrogen which is produced under their influence during 2 hours at 37° at neutral reaction was estimated by the method described in the previous article. The materials necessary for this were of the following description.

As substrate we made use of a solution of nicotine (Nikotin, pur, Merck) in water, of which the titre towards sodium alizarinsulphonate as indicator was determined. For the calculation, the figure thus obtained has to be multiplied by two, since only one nitrogen atom can be titrated, whilst in the decomposition of the nicotine, both N atoms of the nicotine molecule become converted into volatile non-nicotine nitrogen.

The enzyme extracts were prepared as follows:

(*a*) Expressing the juice from freshly gathered and finely chopped tobacco leaves (obtained from the Agricultural School at Mikveh Israel, near Jaffa)[1] and diluting it ten times.

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[1] For which we have to thank the Director, Mr Krause.

(*b*) Preparing extracts from finely powdered dried green tobacco leaves of the same variety as in (*a*); the drying took place in a vacuum drying oven at about 30°.

(*c*) Making macerates from slowly dried powdered tobacco leaves, of a brown colour, as they were supplied from the stores of Messrs Lubliner at Rosh-Pinah in Upper Galilee[1].

(*d*) Preparing macerates from finely cut cured tobacco as used by Messrs Maspero in their cigarette manufactory.

The macerates were prepared by allowing 10 g. of the tobacco together with ten times the quantity of water to stand at room temperature for 6 hours and then filtering.

I. EXPERIMENTS WITH THE EXPRESSED JUICES.

1. *Peptolytic ferment.*

In order to detect effects due to peptolytic ferments 5 cc. of the juice after neutralisation were treated with 10 cc. peptone and 5 cc. disodium hydrogen phosphate as buffer.

The amino N titration was carried out by Sörensen's method and the following values were obtained:

$$t_0 = 13{\cdot}85, \qquad t_2 = 14{\cdot}7, \qquad t_4 = 14{\cdot}8.$$

The control test showed no change.

It is thus evident that a progressive, although slow, hydrolysis of the peptone occurs.

2. *Nicotine-splitting ferment.*

(*a*) The following solution was used:

5 cc. nicotine ($= 6{\cdot}1$ cc. $N/10$ H_2SO_4) + 5 cc. expressed juice + $6{\cdot}1$ cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine showed a titre of 26 cc. $N/10$ H_2SO_4 corresponding to the content of volatile non-nicotine nitrogen.

(*b*) In this case the following mixture was used:

5 cc. nicotine ($= 16{\cdot}2$ cc. $N/10$ H_2SO_4) + 20 cc. expressed juice (made neutral to litmus) + $16{\cdot}2$ cc. $N/10$ H_2SO_4 (for neutralisation).

The solution was made up to 100 cc. and 20 cc. of it taken for titration.

Calculated quantity of nicotine $= 3{\cdot}24$ cc. $N/10$ H_2SO_4. Non-nicotine N in the filtrate from the nicotine precipitate $= 2{\cdot}6$ cc. $N/10$ H_2SO_4.

(*c*) 5 cc. expressed juice contain a quantity of

nicotine + non-nicotine N corresponding to $0{\cdot}28$ cc. $N/10$ H_2SO_4.

Hence, even if the total alkalinity of the juice should be considered as corresponding to non-nicotine nitrogen, there are still in the distillates, after precipitating the nicotine, non-nicotine bases originating from the nicotine corresponding to $(2{\cdot}6 - 0{\cdot}28)$ cc. $= 2{\cdot}32$ cc. $N/10$ H_2SO_4.

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[1] We have to acknowledge our obligations to the Directors of the firm of Lubliner, and especially Dr Zlocisti, for their valuable assistance.

The extent to which the process goes on may be calculated as follows:

We commenced with a quantity of nicotine corresponding to titre value of 3·24 cc. $N/10$ H_2SO_4. This means a total N content corresponding to 6·48 cc. $N/10$ H_2SO_4.

From this there resulted nitrogen products, in the form of volatile bases, corresponding to a titre of 2·32 cc. $N/10$ H_2SO_4.

The process therefore occurs to the extent of 35 %.

II. Experiments with the Macerates.

3. *Nicotine-splitting ferment in macerates from dried, brown, tobacco.*

The following solution was used:

5 cc. nicotine (=15 cc. $N/10$ HCl) + 5 cc. macerate + 15 cc. $N/10$ H_2SO_4 (for neutralisation).

The mixture was made up to 50 cc. and 20 cc. of this taken for the experiment.

Calculated quantity of nicotine	=6 cc. $N/10$ H_2SO_4
Non-nicotine N in the distillate after precipitating the nicotine	=1·9 cc. $N/10$ H_2SO_4

2 cc. of the maceration liquid contain:

(Nicotine + non-nicotine) N	=0·53 cc. $N/10$ H_2SO_4
∴ Volatile non-nicotine N arising from nicotine...	=1·37 cc. $N/10$ H_2SO_4

From a solution of nicotine corresponding to a titre of 6 cc. $N/10$ H_2SO_4 and therefore containing total N equivalent to 12 cc. $N/10$ H_2SO_4, there was thus produced a quantity of non-nicotine bases equivalent to 1·37 cc. $N/10$ H_2SO_4; hence decomposition must have occurred to the extent of 11·4 %.

4. *Comparative investigation of the nicotine-splitting ferments in the macerates obtained from unfermented, dry, green and brown tobacco, and from dried, cured tobacco.*

(a) *Dried green tobacco leaves* (unfermented).

10 g. of dry green tobacco leaf were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 1·5 cc. $N/10$ H_2SO_4; non-nicotine N = 1·3 cc. $N/10$ H_2SO_4.

For the purpose of the test a solution was made up containing:

10 cc. macerate as above + 5 cc. nicotine (=3 cc. $N/10$ H_2SO_4) + 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine contained non-nicotine N corresponding to 1·9 cc. $N/10$ H_2SO_4.

The balance-sheet of the decomposition of the nicotine is therefore as follows:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	=0·75
Nicotine in the substrate	=3·00
Calculated total nicotine	=3·75
Non-nicotine N in the macerate	=0·65
Non-nicotine N in the substrate + macerate	=1·9
Non-nicotine N of substrate, by difference	=1·25

Since from a solution of nicotine, equivalent to 3·75 cc. $N/10$ H_2SO_4, which therefore actually contains nicotine N equivalent to 7·5 cc. $N/10$ H_2SO_4, there was produced non-nicotine N corresponding to 1·25 cc. $N/10$ H_2SO_4, the process has gone on to the extent of 16·7 %.

(*b*) *Dried brown tobacco leaf* (unfermented).

10 g. tobacco were treated with 100 cc. water and then filtered.

20 cc. of macerate contained:

Nicotine N = 1·2 cc. $N/10$ H_2SO_4; non-nicotine N = 1·8 cc. $N/10$ H_2SO_4.

For the test the following solution was used:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate, after precipitation of the nicotine, contained non-nicotine N = 3·0 cc. $N/10$ H_2SO_4.

Thus we have:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	= 0·6
Nicotine in the substrate	= 3·0
Total nicotine	= 3·6
Non-nicotine N in macerate + substrate	= 3·0
Non-nicotine N in the macerate ...	= 0·9
Non-nicotine N in the substrate ...	= 2·1

Thus, in a solution containing nicotine showing a titre for N of 3·6 cc. $N/10$ H_2SO_4 and therefore actually corresponding to a total N content equivalent to 7·2 cc. there was produced non-nicotine N corresponding to 2·1 cc. $N/10$ H_2SO_4; hence the process has gone on to the extent of 29 %.

(*c*) *Fermented tobacco.*

10 g. of this tobacco were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 2·2 cc. $N/10$ H_2SO_4; non-nicotine N = 2·6 cc. $N/10$ H_2SO_4.

The following solution was made up for the test:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine was found to contain non-nicotine N corresponding to 2·0 cc. $N/10$ H_2SO_4.

The data for the nicotine splitting are:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	= 1·1
Nicotine in the substrate	= 3·0
Total nicotine	= 4·1
Non-nicotine N in macerate + substrate	= 2·0
Non-nicotine N in macerate	= 1·3
Non-nicotine N in the substrate ...	= 0·7

Since a solution containing nicotine giving a titre for N of 4·1 cc. $N/10$ H_2SO_4 and therefore actually containing total N equivalent to 8·2 cc. gave rise to non-nicotine N corresponding to 0·7 cc. $N/10$ H_2SO_4, the process has gone on to the extent of 8½ %.

SUMMARY AND DISCUSSION OF RESULTS.

From the experiments described in II. 4. (*a*)–(*c*), which were carried out under similar conditions, it appears that the fermentative effect is greater in the case of unfermented tobacco leaves allowed to dry naturally than in the case of tobacco obtained by drying rapidly. Of course the comparison is not absolute, since the latter may still contain not inconsiderable amounts of water. The effectiveness of an equal amount of fermented tobacco in comparison is remarkably slight.

Thus it seems that the greater part of the activity disappears during the fermentation process. These phenomena remind us very strongly of the results which in the meantime have been observed in this Institute in connection with the alteration in polypeptide-splitting activity during the germination process.

Here also the stability of the enzyme system becomes much reduced.

It will be our object in further investigations to study more definitely the identity of the products of decomposition of the nicotine, as also the rôle of the oxidase effect occurring during the process of fermentation.

We have to acknowledge our thanks to Miss M. Goldberg and Miss S. Beham for their assistance in carrying out the determinations entailed in this work.

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CXXV. CONTRIBUTIONS TO THE STUDY OF BRAIN METABOLISM.

II. CARBOHYDRATE METABOLISM.

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In a previous communication [B. E. and E. G. Holmes, 1925] we were able to show that the "resting" lactic acid content of rabbits' brains was greatly reduced, if the animals had convulsed, or were on the point of convulsing, as a result of a dose of insulin.

That the low values obtained as a result of insulin administration might possibly have been due to an increased oxidation of lactic acid as a consequence of the presence of the insulin, is suggested by the work of Neuberg, Gottschalk and Strauss [1923] who demonstrated an increased formation of acetaldehyde (which might have come from the oxidation of lactic acid) in tissue hashes to which insulin had been added. We found, however, reason to suppose that, although reducing substance can be extracted from rabbits' brains after death, none of it (or at most a very small amount) is glucose. Whilst we found that intact or chopped brain does not by itself give rise to increased lactic acid production on standing at body p_H, we confirmed the statement of Warburg, Posener and Negelein [1924] that it does so if glucose be added to it. The suggestion therefore presented itself that the "resting" lactic acid values obtained for brain were ultimately dependent on the level of the blood-sugar, and not due to a direct effect of insulin, or any accompanying impurity, on the brain cells.

Since our first experiments, we have found it possible to obtain, by suitable means, values for brain lactic acid very considerably higher than any "normal" values, and this without any marked rise of blood-sugar. In spite of this, however, we are inclined to believe that the lowering of the brain lactic acid after insulin is a consequence of the fall in blood-sugar, and does not occur apart from the latter.

It was at first hoped that it would be possible to plot a brain lactic acid—blood-sugar curve. For reasons to be discussed immediately, this could only be done, if at all, by using an unjustifiably large number of animals. It is, however, easy to show that the brain lactic acid does not fall appreciably until the blood-sugar has dropped considerably, whilst, provided that the blood-sugar has fallen below about 70 mg. per 100 cc., the resting lactic acid value is always well below the normal.

The experimental results are arranged in two tables. Table I shows the results obtained from groups of animals from the same litter. (The normal values from animals of the same litter show much less variation than those from animals taken at random.) Table II contains values obtained from animals used for experiments performed with another object, in which the brain lactic acid and the blood-sugar were both determined.

No. 5 β deserves particular attention. The animal was not starved, and was therefore given a large dose of insulin (30 units). Convulsions took place after 98 minutes. After these had lasted 5 minutes 8 cc. of 10 % glucose in normal saline were given subcutaneously. The animal recovered. Twenty minutes later, blood samples were taken, and the animal was killed. The blood-sugar was 119 mg. per 100 cc., while the brain lactic acid was 56 mg. per 100 g. This last value is higher than any obtained for convulsed animals, and is evidently rising towards the normal.

Table I.

Litter no.	Remarks		Dose of insulin (units)	Interval before killing (minutes)	Blood-sugar mg. per 100 cc.	Brain lactic acid ("Resting") mg. per 100 g
Y 1	Normal, starved		—	—	138	95
Y 2	Insulin, starved		20	40	107	88
Y 5	,, ,,		10	—	92	93
Y 3	,, ,,		10	80	65	49
Y 6	,, ,,	C	10	—	68	40
Y 4	,, ,,	C	10	100	—	44
2 α	Insulin, starved		10	63	95	76
4 α	,, ,,	C	10	82	—	29
1 α	,, fed	C	40	422	50	45

"C" = convulsed.

Table II.

No.	Remarks	Blood-sugar mg. per 100 cc.	Resting brain lactic acid mg. per 100 g.
X 1	Insulin	124	96
13 F	Normal	150	101
17 D	,,	131	75
23	Insulin	61	44
5 β	Insulin, convulsed. Recovered with 8 cc. 10 % glucose	119	56

The blood was drawn from an ear vein.
Blood-sugar by the method of Hagedorn and Jensen [1923].
Brain frozen and crushed before weighing [B. E. and E. G. Holmes, 1925].
Lactic acid by method of Meyerhof [1920]. (Uncorrected figures.)

Since it is seldom possible to obtain a litter of more than six rabbits, any single series of values is limited to that number. This makes it extremely difficult to hit off any required blood-sugar level, for the animal must be killed immediately the sample has been taken, and long before the sugar estimation can be completed.

It will be observed that the lactic acid does not fall until the blood-sugar is below about 70 mg. per 100 cc. Since values ranging from 100–75 mg. per 100 g. (we have had two values as low as 65) are obtained for the resting brain lactic acid of normal animals from different litters, it is hardly to be expected than an exact correlation between blood-sugar and brain lactic acid could be found above this level.

The fact that, for instance, rabbit "Y 2" (Table I) showed no appreciable fall in brain lactic acid 40 minutes after 10 units of insulin, shows that insulin, of itself, exerts no direct effect on the brain lactic acid metabolism.

It is, of course, certain that there are many factors complicating the issue. If the amount of lactic acid formed depends upon the amount of glucose made available *via* the blood stream, it must vary, not only with the level of the blood-sugar, but also with the rate of blood supply to the brain, and, further, by the rate at which glucose is able to diffuse into the cell, and reach the point at which chemical change occurs. Of these three factors, one only is known, namely, the blood-sugar level.

Apart from these considerations, Hiller and collaborators have recently shown [Hiller, Almer, Linder and Van Slyke, 1925] that only a part of the reducing substance, estimated in blood by the methods in general use, is glucose. A variable but not inconsiderable part of it appears to be some material or materials which is not fermented by yeast, nor destroyed by the glycolytic enzyme of the blood itself. In fact, they suggested that after insulin the glucose content of rabbits' blood may fall to zero, though a very definite reducing power still remains. If these findings be correct, it is hardly likely that the figures appearing in our tables under "blood-sugar" represent at all accurately the glucose content of the blood.

We feel, however, that the experiments here described make it improbable that the fall in brain lactic acid which we observe is due to an immediate effect of the insulin, or of any substance injected along with it, on the brain cells. In the latter connection, it is worth mentioning that in our earlier experiments we used a dried insulin preparation (supplied by Messrs Boots); in those here described, we employed a liquid preparation prepared by Burroughs and Wellcome for clinical use.

SUMMARY.

A comparison of the blood-sugar values, and the resting lactic acid content of the brains of rabbits, shows that the fall in the latter resulting from the injection of insulin does not occur until the blood-sugar has reached a fairly low level. At the convulsive or flaccid (pre-convulsive) stage the brain lactic acid content is very small. One experiment indicates that the lactic acid content rises again when the animal has recovered as the result of injection of glucose.

From these results it appears probable that the fall in the resting lactic acid content of the brain after insulin injection is not due to a direct effect of insulin in promoting increased oxidation of lactic acid, nor to any direct effect of insulin or an accompanying impurity in depressing the production of lactic acid by the brain cells, but is rather caused by the fall in the blood-sugar level, and the resulting shortage of glucose in the brain.

One of us (B. E. H.) is in receipt of a grant from the Medical Research Council.

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CXXVI. THE ESTIMATION OF FAT IN BLOOD.

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In view of the large number of pathological conditions in which the fat content of the blood is increased, and of our relatively meagre knowledge of fat transport and metabolism, it is eminently desirable than an accurate, and, as far as possible, non-subjective method be devised for the estimation of fatty acids in blood. The methods at present available fall into two classes: first, nephelometric, of which that of Bloor [1914] is typical; second, oxidative, of which those of Gade [1919] and Bang [1918] are typical.

Nephelometric methods are open to several objections. The personal factor is comparatively great, and the time factor is important since the suspension of fat flocculates at a measurable and varying rate [Blix, 1924]. Moreover, Bloor's method estimates both cholesterol and fatty acids, the latter being ultimately determined by difference. This last objection, of course, does not extend to the more recent method of Bloor, Pelkan and Allen [1922].

In Gade's method [1919] the fat extracted from the dried blood by ether is allowed to react for a definite time with an excess of potassium permanganate, the excess being then determined by the addition of excess of oxalic acid and back-titration with permanganate. The method is somewhat cumbersome, the reaction with permanganate must be carefully controlled with regard both to time and temperature, and an empirically determined correction has to be applied. Bang's method [1918], in which the fat is oxidised by chromic acid, the excess being determined by allowing it to act on potassium iodide and titrating the liberated iodine with thiosulphate, gave good results in the hands of Gade, as reported in his comprehensive review [1919]. As in the case of Bloor's method, however, it gives both cholesterol and fat, and the true fat content is obtained by estimating the cholesterol separately and subtracting.

Hitherto, the small amount of fatty acid obtainable from the 2 cc. of blood usually available for routine estimations has rendered it impossible to utilise, with any degree of accuracy, the hydrolytic method of estimation in which the fat is saponified by sodium hydroxide and the excess alkali titrated with acid. The new micro-burette described by Rehberg [1925], however, delivers

0·10 cc. of solution and can be used for titrations requiring fractions of that amount with at least as great accuracy as is attainable with the 10 cc. of the macro-titration. Since 0·10 cc. of $N/10$ NaOH is equivalent to 26·86 mg. of tripalmitin or 29·67 mg. of tristearin, the burette can evidently be used to titrate the fatty acids liberated by hydrolysis from the fat contained in only 1·0 cc. of normal blood.

Acting on these considerations, we attempted to estimate known amounts of synthetic tripalmitin. This was prepared from pure palmitic acid and glycerol by the method of Scheij [1899], purified by repeated extraction with alcohol, and many recrystallisations from alcohol-ether mixture, and finally from pure ether. Quantities were taken corresponding to the amounts present in 1·0 cc. of normal and lipaemic bloods (3–18 mg.). For this purpose the fat was dissolved in pure anhydrous ether and the requisite quantities of the solution were measured into 50 cc. round-bottomed flasks. After evaporation of the ether, 5 cc. of $N/10$ NaOH and 5 cc. of absolute alcohol were added, and the mixture allowed to evaporate almost to dryness on the water-bath, a process which occupied about 2 hours. We found it advisable at this stage not to attempt the direct titration of the excess of sodium hydroxide, for during the manipulation before and after hydrolysis a small amount of carbon dioxide is inevitably absorbed. Since the volumes involved in the titration are so small, and an indicator such as phenolphthalein must be used, this introduces a serious error. After hydrolysis we therefore added to the mixture 5 cc. of $N/10$ HCl. This exactly neutralised the whole of the sodium hydroxide, and liberated the fatty acids which had been produced during the hydrolysis. We then boiled down to about 1 cc. in order to drive off the absorbed carbon dioxide. The residual liquid was transferred quantitatively to a 10 cc. volumetric flask, absolute alcohol being used for the washings and for making up the volume to 10 cc. 1 cc. of this final solution, corresponding to one-tenth of the amount of fat originally taken, was titrated with $N/10$ sodium hydroxide from the Rehberg burette. The sodium hydroxide used in the titration must, of course, be free from carbonate. In our experiments, it was kept in a paraffined bottle provided with a syphon and tap, the air inlet being guarded by a soda-lime tower. In filling the burette, a short rubber tube connected the burette nozzle and the syphon. In this way the standard NaOH was prevented from coming into contact with the air. The indicator used was phenolphthalein, and a correction was applied for the amount of indicator, volume of fluid, etc. as described by Rehberg [1925].

It cannot be over-emphasised that for these estimations both reagents and apparatus must be accurately standardised. Thus, if the hydrochloric acid be slightly below $N/10$, or if slightly less than 5·0 cc. be added, the fatty acids will be incompletely liberated. Conversely, if acid be added in slight excess, it will remain to be neutralised in the titration. In either case, apparently slight variations may lead to serious errors, notwithstanding the subsequent dilution, since the volume of the titrating fluid is so small. Careful manipulation is necessary at every stage, but granted this, as our results abundantly show (Table I), not only do duplicate titrations from the same sample agree, but duplicate samples, treated separately throughout, give results within 5 % of the theoretical. We may point out that in several of the duplicate results recorded, one of the estimations has been carried out by each of us. An important advantage of the method is that in case of necessity, as many as eight or nine titrations can be made on each sample.

Table I. *Estimation of fat in pure solutions of tripalmitin.*

Fat present g. per 100 cc.	Titre (corrected)*	Fat found g. per 100 cc.	Error %
0·300	10·3		
	10·8	0·283	5·6
	12·0		
	11·3	0·310	3·3
0·600	20·5		
	20·4	0·596	0·6
	22·8	0·618	3·0
	23·2		
0·800	29·8		
	29·8	0·800	0·0
0·900	34·0		
	34·6	0·921	2·3
1·200	43·8		
	43·6	1·174	2·2
	43·8		
	44·4	1·185	1·2
1·500	53·9		
	53·7	1·445	3·6
1·800	68·9		
	70·0	1·765	2·0
	69·6		
	69·8	1·770	1·7

* The correction derived from macro- and micro-titration of the acid and alkali used, with a standard amount of titration fluid (1 cc.) and a standard amount of indicator, was 0·7.

In extending the method to blood, we have not concerned ourselves with the selective extraction by different solvents of the various constituents of the blood-fat, but have contented ourselves with showing its applicability to the estimation of the total fatty acids of the blood irrespective of the state of combination in which they occur. 2·0 cc. of blood were added with constant shaking to about 30 cc. of alcohol-ether mixture (three parts alcohol, one part ether) in a 50 cc. flask. The mixture was brought just to boiling point, cooled, and made up to 50 cc. with alcohol-ether. After thorough mixing, it was allowed to settle and 25 cc. of the clear supernatant liquid were withdrawn for analysis. This procedure was adopted to avoid the concentration of the extract which may take place, to a varying degree, during filtration. The remainder of the procedure was exactly as described for pure solutions of fat. Table II gives some of the results we have obtained in estimating fat in blood, alone, and with added fat.

Notwithstanding the agreement of duplicate analyses, there still remains the possibility that the extract may contain, in addition to fat and fatty acids, some acidic substances soluble in aqueous alcohol-ether mixtures as well as in water. In this category would be included such substances as acetoacetic acid, hydroxybutyric acid, etc. These, though present only in traces in normal blood, may occur to an appreciable extent in pathological specimens. They are, however, of low boiling point, and are, moreover, volatile in steam, so that they are removed by the boiling down after the addition of the hydrochloric acid. To ensure that interfering substances are absent, or are present in negligible quantities if at all, we carried out the following experiment. 50 cc. of the alcohol-ether extract from blood sample No. I (Table II), which was a pathological specimen, were evaporated to dryness on the water-bath. The residue was extracted with two 1 cc. portions of distilled water. The aqueous extract, after filtration, was boiled, and titrated with $N/10$ NaOH from the Rehberg burette. The extract was acid to phenolphthalein, indicating the absence of basic interfering substances, but was rendered distinctly alkaline by the addition of 0·004 cc. of $N/10$ NaOH. We may therefore conclude that the alcohol-ether extract contained no interfering acidic substances.

Table II.

No. of blood sample	Fat added g./100 cc.	Found g./100 cc.	Fat content of blood g./100 cc.	Mean	Error %
I. Ox blood	0·900	2·083	1·183	—	1·5
	1·500	2·698	1·198	—	—
	1·500	2·703	1·203	—	0·5
	1·500	2·706	1·206	1·198	0·7
II. Mixed specimen of human blood —pathological	—	1·845	1·845	—	0·5
	—	1·821	1·821	—	2·0
	—	1·872	1·872	—	1·0
	—	1·877	1·877	1·854	1·0
	0·300	2·130	1·830	—	1·5
	0·300	2·122	1·822	—	2·0
	1·200	3·073	1·873	—	1·0
	1·500	3·427	1·927	—	4·0
III. Human blood—normal	—	0·306	0·306	0·312	1·8
	—	0·317	0·317	—	1·8

In this table the fat has been calculated as tripalmitin.

We have attempted to modify the method so as to permit of using much smaller quantities of blood. Even with careful technique, however, the errors in measuring with the ordinary pipette are sufficient to vitiate the results. By substituting for pipettes measuring instruments based on the principle of the Rehberg burette, it would, no doubt, be possible to obtain moderately accurate results with quantities of blood less than 0·5 cc. The method, even as we have used it, calls for very careful work, and can hardly be used as a routine method by the busy technician; it is primarily a method for research. However, the use of a modified Rehberg burette for the measurement of the alkali used in hydrolysis and the acid subsequently added renders the method less delicate and more suitable for routine use.

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CXXVII. A SYNTHESIS OF *N*-METHYL-PUTRESCINE AND OF PUTRESCINE.

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In connection with other work proceeding in this laboratory it became necessary to prepare the hitherto unknown *N*-methylputrescine, and it was hoped that this compound would result from the action of methylamine on δ-bromobutylamine thus; $Br(CH_2)_4NH_2 \xrightarrow{CH_3NH_2} CH_3.NH.(CH_2)_4.NH_2$. Unfortunately, under all experimental conditions tried, the effect of methylamine on this and the corresponding iodo-compound was similar to that of sodium hydroxide [von Braun and Beschke, 1906; Gabriel, 1909], ring closure occurring with exclusive formation of pyrrolidine.

It was found, however, that δ-iodo- (and δ-bromo-) butylamine can be benzoylated almost quantitatively and that the monobenzoyl-δ-iodobutylamine obtained can then be converted by the action of methylamine into monobenzoyl-*N*-methylputrescine, from which the base itself results on hydrolysis with hydrochloric acid.

$NH_2.(CH_2)_4.I \longrightarrow C_6H_5.CO.NH.(CH_2)_4.I \longrightarrow C_6H_5.CO.NH.(CH_2)_4.NH.CH_3 \longrightarrow NH_2.(CH_2)_4.NH.CH_3$.

By the use of ammonia in place of methylamine, putrescine itself can be prepared, and this synthesis may prove useful to others, as it did to us, on account of the difficulty experienced in obtaining pyrrole which is the starting point of the usual synthesis due to Ciamician and Zanetti [1889, 1890] and Willstätter and Heubner [1907]. The synthesis described in this paper has the further advantage that it involves the preparation of the hitherto undescribed *mono*benzoylputrescine which is inaccessible from other directions and may be useful in further synthetic work.

The secondary base di-δ-benzoylaminobutylamine

$$NH[(CH_2)_4.NH.CO.C_6H_5]_2$$

is also formed during the interaction of ammonia and benzoyl-δ-iodobutylamine; the base di-δ-aminobutylamine has been prepared from this by hydrolysis and is described in the experimental section.

N-Methylputrescine has also been synthesised in the following way:

$$\mathrm{Br.(CH_2)_3.OC_6H_5} \xrightarrow{\mathrm{CH_3NH_2}} \mathrm{CH_3.NH.(CH_2)_3.OC_6H_5} \xrightarrow{\mathrm{HBr}}$$

$$\mathrm{CH_3.NH.(CH_2)_3.Br.} \xrightarrow{\mathrm{C_6H_5.COCl}} \mathrm{CH_3.N.CO.C_6H_5.(CH_2)_3Br} \xrightarrow{\mathrm{KCN}}$$

$$\mathrm{CH_3.N.CO.C_6H_5.(CH_2)_3.CN} \xrightarrow{\mathrm{Na+C_2H_5OH}} \mathrm{CH_3.NH.(CH_2)_4.NH_2}.$$

Difficulties were, however, encountered in the preparation of the nitrile, and the best experimental conditions for the synthesis were not studied in detail.

EXPERIMENTAL.

δ-Bromo- and δ-iodobutylamine were prepared from α-γ-dibromopropane according to the directions of von Braun and Beschke [1906] and Gabriel [1909].

The hydrochloride and picrate of phenoxybutylamine, one of the intermediate substances in the preparation, melt at 157·5° (corr.) and 159·5° (corr.) respectively.

Benzoyl-δ-bromo- and benzoyl-δ-iodobutylamine. δ-Bromo- and δ-iodobutylamine are benzoylated by adding 2 mols. benzoyl chloride to an aqueous solution of the amine halide cooled in ice. Solid sodium bicarbonate is then gradually added with constant shaking, the liquid being kept at 0°. The solid benzoyl derivative is extracted with ether, washed with dilute sulphuric acid and dilute potash and dried over potassium carbonate. Evaporation of the ether leaves colourless needles. Benzoyl-δ-bromobutylamine recrystallised from ether melts at 52° (corr.). Benzoyl-δ-iodobutylamine agrees in its properties with that prepared by von Braun and Beschke [1906] from benzoylpyrrolidine. Yield 90 %.

Monobenzoyl-N-*methylputrescine.* 13 g. benzoyl-δ-iodobutylamine are dissolved in 60 cc. absolute alcohol and boiled gently under a reflux condenser with 30 cc. 33 % alcoholic methylamine for 2 hours. The alcohol and methylamine are removed *in vacuo* on the water-bath. The resulting syrup is dissolved in water and the base liberated with NaOH. The insoluble oil is extracted with chloroform. Evaporation of the dried extract leaves a pale oil insoluble in water and sparingly soluble in ether, but readily soluble in alcohol. The addition of HCl gives a syrupy hydrochloride which can be precipitated from alcoholic solution by ether in colourless needles melting at 150°. On benzoylation in the ordinary way the base gives dibenzoyl-*N*-methylputrescine.

N-*Methylputrescine dihydrochloride.* The syrupy monobenzoyl-*N*-methylputrescine is hydrolysed by heating it at 130° for 8 hours in a sealed tube with five times its weight of concentrated HCl. The hydrochloride may not crystallise at once but comes out on standing with absolute alcohol for a day. Yield 70 %, calculated from benzoyliodobutylamine. The hydrochloride is very soluble in water but insoluble in alcohol and acetone. Recrystallised from 95 % alcohol it forms hygroscopic needles melting at 179° (corr.).

Analysis.

	Found %	Calculated for $C_5H_{14}N_2.2HCl$ %
C	34·11	34·29
H	8·86	9·14
N	15·99	16·00
Cl	40·52	40·57

N-*Methylputrescine*, liberated from aqueous solution of the hydrochloride by means of alkali, can be extracted with chloroform and is a colourless oil boiling at 161–163°, very soluble in water and almost insoluble in ether. It has a strong smell, similar to piperidine but not so pungent and fumes in moist air. The *dipicrate*, recrystallised from water, forms prisms or needles melting with decomposition at 229–230·5° (corr.). It is almost insoluble in alcohol and cold water.

Analysis. Picric acid: found (by nitron) 81·78 %; calc. for

$C_5H_{14}N_2.2C_6H_3O_7N_3$ 81·79 %.

The *chloroaurate*, recrystallised from *N*/10 HCl, forms deep yellow prisms, containing 1 mol. water of crystallisation, melting at 192° (corr.) with previous softening, and decomposing at 215° (corr.).

Analysis. Au: found 49·24 %; calc. for $C_5H_{14}N_2.2HAuCl_4.H_2O$ 49·27 %. After drying between 100 and 110°, Au: found 50·53 %; calc. for

$C_5H_{14}N_2.2HAuCl_4$ 50·41 %.

H_2O: found 2·55 %; calc. for $1H_2O$ 2·25 %. (Slight loss of HCl.)

The *chloroplatinate*, recrystallised from dilute HCl, forms reddish prisms decomposing at 230·5° (corr.). It can be precipitated from aqueous solution as a crystalline powder by alcohol.

Analysis. Pt: found 38·01 %; calc. for $C_5H_{14}N_2.H_2PtCl_6$ 38·10 %.

The *picrolonate*, recrystallised from water, forms needles decomposing between 254° and 265° according to the rate of heating. It is very slightly soluble in alcohol.

The *mercuric chloride compound* is precipitated from alcoholic solution in needles changing to plates. Recrystallised from water it melts at 148° (corr.). It is sparingly soluble in alcohol.

The *dibenzoyl compound* is very soluble in chloroform and alcohol. It is precipitated from chloroform by ether in small needles, and larger needles are obtained by slow crystallisation from dilute alcohol. M.P. 115·5° (corr.).

Monobenzoylputrescine. 10 g. benzoyl-δ-iodobutylamine are heated in a sealed tube with 6 g. ammonia in 50 cc. alcohol for 4 hours at 100°. The alcohol and excess ammonia are removed *in vacuo* on the water-bath. The resulting oil is stirred up with warm dilute HCl and shaken with ether. The aqueous layer is contaminated with an ether-insoluble oil which must be entirely removed by repeated filtration. The colourless solution thus obtained is made alkaline with NaOH and extracted with chloroform. Evaporation of the dry extract gives a pale oil which rapidly absorbs CO_2. On dissolving this oil in the requisite amount of 10 % HCl to give a neutral solution, a hydrochloride gradually comes down as a white crystalline powder. The deposition of this salt is accelerated by immersing the solution in a freezing mixture. This is di-δ-benzoylaminobutylamine hydrochloride and is removed by filtration when separation is complete. The filtrate can be treated with benzoyl chloride and NaOH in the usual manner when dibenzoylputrescine immediately separates as a white solid which can be filtered off and hydrolysed, giving putrescine hydrochloride. Yield of dibenzoylputrescine 50 %. For the isolation of *monobenzoylputrescine hydrochloride* the filtrate from the di-δ-benzoylaminobutylamine hydrochloride is concentrated and poured into 800 cc. acetone when monobenzoylputrescine hydrochloride separates in needles. Yield about 40 %. The remainder can be isolated by repeating the acetone treatment several times. Monobenzoylputrescine hydrochloride is soluble in water and alcohol. Recrystallised from alcohol, it melts at 171° (corr.).

Analysis. Cl: found 15·40 %; calc. for $C_{11}H_{17}ON_2Cl$ 15·51 %.

The base is an oil slightly soluble in water, insoluble in ether but very soluble in chloroform and alcohol.

The *picrate* forms prisms sparingly soluble in water and alcohol. Recrystallised from water it melts at 173° (corr.).

Putrescine dihydrochloride. Monobenzoyl- (or dibenzoyl-) putrescine is hydrolysed by heating in a sealed tube for 8 hours at 130° with five times its weight of concentrated HCl. The product is dissolved in boiling water and cooled. Benzoic acid is filtered off and the filtrate extracted with ether to remove the remainder. The aqueous layer is evaporated on the water-bath, leaving a white crystalline mass. This is washed on to a filter with a little absolute alcohol. Yield of putrescine hydrochloride (M.P. 300°) from crude dibenzoyl compound 73 %. A further 11 % can be recovered from the filtrate by evaporating and re-treating with alcohol. The yield from pure monobenzoylputrescine hydrochloride is theoretical.

From the hydrochloride the picrate, chloroaurate and chloroplatinate were prepared. The melting points agreed with those given in the literature.

Analyses. Chloroaurate. The salt lost 2 mols. H_2O on drying at 95°. Au: found 51·29 %; calc. for $C_4H_{12}N_2.2HAuCl_4$ 51·34 %. *Chloroplatinate.* Pt: found 38·99 %; calc. for $C_4H_{12}N_2.H_2PtCl_6$ 39·19 %.

Di-δ-benzoylaminobutylamine hydrochloride, recrystallised from alcohol, forms prisms, M.P. 233° (corr.). When mixed with monobenzoylputrescine hydrochloride the melting point is depressed to 171°, the melting point of pure monobenzoylputrescine hydrochloride. It is sparingly soluble in cold water and alcohol. Yield 10 %.

Analysis. Cl: found 8·81 %; calc. for $C_{22}H_{30}O_2N_3Cl$ 8·78 %.

The *base* is precipitated from a solution of the hydrochloride by addition of sodium hydroxide solution in needles melting at 68°. It is very soluble in alcohol and chloroform but sparingly in water and ether. It is purified by precipitation from alcohol by water.

The *picrate*, recrystallised from very dilute acetone, forms needles melting at 121° (corr.). It is sparingly soluble in water and alcohol.

Di-δ-aminobutylamine hydrochloride is obtained by hydrolysis of the dibenzoyl compound with HCl under the conditions employed for the hydrolysis of monobenzoylputrescine hydrochloride. It is very soluble in water but insoluble in alcohol. Recrystallised from dilute alcohol it forms needles softening indefinitely at about 290° with blackening.

The *base* is an oil smelling like putrescine and fuming in moist air. It is very soluble in water and chloroform. It is not attacked by boiling 25 % NaOH and is not volatile in steam.

The *tripicrate* forms long thin plates, often resembling needles, melting with decomposition at 255° (corr.) after recrystallisation from water. It is sparingly soluble in alcohol.

Analysis. Picric acid: found (by precipitation with nitron) 81·37 %; calc. for $C_8H_{21}N_3 . 3C_6H_3O_7N_3$ 81·19 %.

The *chloroaurate*, recrystallised from *N*/10 HCl, forms prisms melting at 209° (corr.) with decomposition.

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CXXVIII. A NEW HYDROLYSIS PRODUCT FROM ELASTIN.

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It is generally believed that Millon's reaction obtained with proteins is due to the presence of tyrosine. However, such a substance as gelatin also gives this reaction, although no tyrosine is formed upon hydrolysis. Similar conditions prevail in the case of elastin, which in its chemical composition shows some relation to gelatin; no tyrosine could be isolated from the crystallised crude leucine originally obtained from elastin in a joint investigation by W. Biehler and myself[1]. Upon examination of another sample of elastin only such a small amount as 0·15 % of crude tyrosine could be found, which hardly explains the strong Millon reaction obtained with the original material.

It occurred to the author therefore that, in addition to tyrosine, other substances, thus far unknown as decomposition products, also give Millon's reaction. This suspicion was fully verified in the case of elastin. First, it was shown that the basic portion, precipitated from the hydrolysis products by phosphotungstic acid, gave the reaction, even when the precipitate was treated in a manner which precluded any retention of the filtrate. The substance yielding the reaction possesses therefore pronounced basic properties. It was isolated in the following manner.

Air-dry elastin was boiled for 5 hours with about five times the quantity of concentrated hydrochloric acid, and the liquid evaporated upon the water-bath to a syrupy consistence. This was diluted with sufficient water to bring the weight of the liquid to about eight times the weight of elastin used. 50 % phosphotungstic acid was then added and the precipitate after standing for several hours carefully washed or triturated with 5 % sulphuric acid.

This precipitate, as stated in greater detail in the above-mentioned communication[1], was treated with lead hydroxide and after careful washing decomposed with lukewarm baryta solution in the well-known manner. Overheating or great excess of baryta was carefully avoided by adding the baryta solution very slowly in small portions and removing it by suction, when the reaction had remained alkaline for several hours. The residue was treated twice in the same manner. The decomposition took about 2 days and the solution of the basic substances contained only traces of excess of baryta as well as some lead. The lead, after addition of animal charcoal, was removed with hydrogen sulphide, and the filtrate was evaporated *in vacuo* to a thin syrup, then boiled with copper hydroxide and filtered. The filtrate was again greatly concentrated, mixed with methyl alcohol until a strong turbidity occurred and set aside at 0° for several days. A considerable precipitate was then noted, partly crystalline, partly consisting of brown flakes. These flakes, on account of their low specific gravity, could mostly be washed off with ice-cold water. The deep blue residue, very slightly soluble in water, was suspended in a little water, and freed from copper by passing hydrogen sulphide through the heated liquid. Upon concentration of the slightly coloured filtrate, long white needles were obtained which were recrystallised from dilute methyl alcohol.

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[1] R. Engeland and W. Biehler, "New Decomposition Products of Elastin," to be published shortly.

2·782 mg. subst. : 5·997 mg. CO_2 and 1·901 mg. H_2O
2·470 mg. „ : 5·328 mg. CO_2 and 1·536 mg. H_2O
3·426 mg. „ : 0·277 cc. N at 708 mm. 11°
Calculated for $C_{16}H_{24}O_5N_2$: C=59·22 %, H=7·46 %, N=8·64 %
Found C=58·83 %, H=7·65 %, N=9·06 %
58·85 %, 6·96 %

The values calculated for formulae with 15 C-atoms would also be very close to those found:

Calculated for $C_{15}H_{22}O_5N_2$: C=58·03 %, H=7·15 %, N=8·41 %
$C_{15}H_{20}O_5N_2$ C=58·41 %, H=6·54 %, N=9·09 %

However the values found for the copper salt strongly suggest the formula with 16 carbon-atoms. The copper salt forms small, dark blue needles, which are very sparingly soluble in water. The crystals purified by crystallisation from hot water gave the following analysis:

(1) 4·524 mg. subst. : 0·950 mg. CuO
(2) 3·388 mg. „ : 0·691 mg. CuO
4·350 mg. „ : 7·875 mg. CO_2 and 2·240 mg. H_2O*
3·960 mg. „ : 0·275 cc. at 723 mm. and 18°*
Found: Cu=16·78 %, C=49·39 %, H=5·76 %, N=7·75 %
16·30 %
Calculated for $C_{16}H_{22}O_5N_2Cu$: Cu=16·48 %, C=49·74 %, H=5 75 %, N=7·38%

* These two analyses were kindly carried out by Dr F. Reindl.

The free substance tastes slightly bitter, and is rather readily soluble in water as well as in acetic (glacial) acid. The aqueous solution upon evaporation on the water-bath is coloured slightly brownish and gives off a flower-like, very characteristic odour. Upon treatment with oxidising agents such as $NaClO_3$ and HCl this odour may be obtained from very small quantities. Upon boiling with concentrated nitric acid a slight yellow coloration is observed, which is intensified when ammonia is added in excess. Millon's reagent produces even in the cold a pink colour, which however is not so intense as with tyrosine. With diazobenzenesulphonic acid in alkaline (Na_2CO_3) solution an orange coloration is observed. An infusion of the conidiophores of two different forms of *Russula* rapidly colours the solution, in presence of air, red with a blue tinge. After several hours the colour changes to bluish brown. The intensity of this colour is seemingly more pronounced than that obtained in the same way with tyrosine. By phosphotungstic acid the substance is precipitated even from very dilute solution. Upon heating in the melting point apparatus the substance begins to be discoloured at about 200° and sinters at about 220°.

The substance was isolated by the same method from two different hydrolyses. I consider the substance isolated, therefore, as a constant hydrolysis product of elastin and call it *hyphasmine* (from τὸ ὕφασμα = the tissue).

The yield amounted to almost 1 %, when very carefully purified elastin was used, and was very much less when another less purified product was examined. Inasmuch as the solution of the substance is decomposed upon heating, it is certain that a considerable portion, originally present, is lost through the method of preparation described above.

With the same method I have tested another material, namely, the membrane of hen's eggs, which also upon hydrolysis yields no tyrosine. However, the substance could not be isolated, nor could I isolate the two new hydrolysis products which were obtained in the investigation jointly carried out with W. Biehler and referred to above.

Concerning the constitution of hyphasmine, one could be tempted to think that we have here a dipeptide, and that tyrosine takes part in its formation. The second component would thus be a hitherto unknown amino-acid—and the compound be characterised by a peculiarly strong resistance to hydrolysis. The composition of the copper salt, however, seems to speak against such an assumption.

CXXIX. OBSERVATIONS UPON THE PREPARATION AND STANDARDISATION OF THE OVARIAN HORMONE.

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(*Received July 31st, 1925.*)

Attempts to demonstrate the presence of an active principle in the ovary date back to the end of the last century, but the first real advance was made by Adler [1912], who published an account of the production of artificial oestrus in animals by the injection of aqueous extracts of whole ovaries. This investigation is entitled to special notice, since it was the first in which a definite attempt at standardisation was made. Adler checked his results by histological examination of the uterine mucosa of his test animals. This work was followed by a series of more elaborate chemical investigations. Iscovesco [1912] extracted whole ovaries with alcohol and obtained a fatty substance possessing activity. Fellner [1913] demonstrated that the active principle was thermostable and soluble in alcohol, ether and acetone.

Very similar results were obtained by Seitz, Wintz and Fingerhut [1914], and later by Herrmann and Fränkel [1915]. The method of the latter workers consists in extraction of the ovaries with alcohol. The solid material is filtered off, and the resulting solution is evaporated to dryness. The residue is extracted with ether, and acetone is added to precipitate the lipoid fraction. The active principle remains after the solvent has been removed by distillation. As much cholesterol as possible is separated from it by fractional crystallisation, after which it is stated that the material can be distilled *in vacuo* at 190°.

Recently the matter has been re-opened by Allen and Doisy [1923], who applied the vaginal smear method of ascertaining the onset of oestrus. Their method for the preparation of the active material is similar to that described by Herrmann and Fränkel. They were able to prepare active material from liquor folliculi, whole ovaries and placenta, but not from corpus luteum. They lay great stress upon the use of liquor folliculi, and appear to incline to the view that the active principle is located mainly in this fluid. In view of the importance of these observations, it was thought that an investigation of these earlier experiments would be of interest.

Method of preparation.

This consists of slight modifications of those employed by Herrmann and Fränkel, and Allen and Doisy. Pigs' ovaries were used as the main source of supply.

The minced tissues were extracted with twice their weight of alcohol, care being taken not to lose any fluid during mincing. After stirring, the mixture was pressed out in a hand press through jean, and the solid material was then extracted in a Soxhlet apparatus with alcohol for 6–9 hours. The alcoholic extracts were then combined and filtered through Chardin papers.

The filtrate was evaporated almost to dryness *in vacuo*, and the residue was taken up in a small volume of alcohol and filtered. On standing, a precipitate of fats and cholesterol separated which was removed by filtration. The solution was again taken down to dryness, and the residue was dissolved in a small quantity of ether, and twice its volume of acetone was added. The precipitated lipins were filtered off. The solid material was then redissolved in ether and again precipitated with acetone. This process was repeated several times. The filtrates were then combined, and evaporated to dryness. The residue was dissolved in a small quantity of alcohol, and some cholesterol was removed by freezing. By repeating this process, the greater part of the cholesterol can be removed. The final product is obtained by distilling off the alcohol *in vacuo*. Some experiments were tried with alternative methods of extraction. The following is an example.

Aqueous extraction.

A small yield of active substance was obtained by mincing 2·78 kg. ovaries (pig) and washing thoroughly with 2·8 l. water. This washing was repeated once more with the same volume of water and the combined washings were acidified until the reddish colour of the washings changed to grey. The washings were concentrated *in vacuo* in a bath at 65° until the volume was approximately 150 cc. Two volumes of alcohol were added and then dilute sodium hydroxide solution until the maximum precipitation occurred. The solution was filtered and the filtrate was once more concentrated to dryness. The solid matter was taken up in hot 95 % alcohol, the solution was filtered and lipins were precipitated by the addition of two volumes of acetone. The precipitate was filtered off and the liquid concentrated. The yield was 4·4 g., the rat-unit of which was 150 mg., corresponding to about nine rat-units from 1000 g. ovaries.

Yields.

The method of preparation already described has been applied to a series of tissues. The ovaries were not specially selected, and were therefore obtained from animals in various stages of the oestrous cycle.

In the following table the yields obtained can be seen.

Table I.

Source	No. of animals	Weight of tissue	Yield g.	Rat unit mg.	Rat unit per kg.	Notes
Pig ovaries, whole ovaries including liquor folliculi	148	1050 g.	1·05	75	14	Without Soxhlet extraction
	70	960	1·14	80	14	
With Soxhlet extraction:						
	92	720	3·69	15	340	
	218	2100	28·8	150	91	
	280	4650	33·9	100	73	
	240	2290	36·0	200	80	
	800	6960	30·35 crude	50	87	Ovaries had very small follicles
			15·85 purified	30	70	
	228	2140	7·6	50	70	
	290	5410	17·75	50	65	
Liquor folliculi only (pig)		250 cc.	0·4	16	100	
		720 cc.	3·7	25	200	
		120 cc.	1·20	50	200	Four ovaries only each with large follicles
Tissue only from above		50 g.	2·25	200	255	
Human ovarian cyst		1100 cc.	Trace	Inactive		
Placenta (human)		385 g.	3·1 crude	200	50	
			0·45 purified	15	85	
Dried pig ovaries		200 g.	13·0	350	150	Dried *in vacuo* at 40°

The yields are similar to those obtained by Allen, Doisy and their co-workers. We have been unable, however, to secure the large yields from liquor folliculi, and from human placenta, described by these workers. The yields obtained by us from liquor folliculi and from the residual ovarian tissue are of the same order. Our findings, therefore, do not support the contention that the active principle is mainly confined to the liquor folliculi. Of a series of commercial preparations tested all proved inactive. One specimen of whole ovaries, dried at 40°, was found to yield 150 rat-units per kg. by the above method of extraction. No active principle could be obtained from hen's eggs. As a negative control, 1 kg. pancreas was worked up by the process. No active material resulted.

Chemical properties.

The active principle consists of a clear, light brown oil, readily soluble in alcohol, ether, acetone, and olive oil. It can be heated to 200° without loss of activity. It withstands saponification only to a limited extent, as can be seen from the following experiments.

1 g. sodium was dissolved in 20 cc. absolute alcohol, and the solution added to 10 cc. of an alcoholic solution of the active principle containing 6·28 g., the rat-unit being 40 mg. The mixture was heated on a boiling water-bath, in an atmosphere of hydrogen for 40 minutes. After cooling, the contents of the flask were poured on to powdered ice, and the resulting liquid was thoroughly extracted with ether. The extracts yielded 0·4 g., which was found to give a positive reaction in rats in doses of 40 mg. The fatty acids obtained

by acidification of the aqueous layer were found to be inactive physiologically, even in doses of 0·5 g. In another experiment, where the reaction was allowed to proceed for 3 hours, the product was quite inactive. It appears therefore that the activity is slowly destroyed under these conditions. Attempts at distillation have up to the present been unsuccessful, owing to excessive frothing.

Purification by digitonin.

The cholesterol-containing substance obtained by the usual alcoholic extraction process (3 g. = 37 rat-units) was dissolved in 60 cc. hot 95 % alcohol. A solution of digitonin (1 g.) in hot 90 % alcohol (100 cc.) was added, and after 1 hour the fine crystalline precipitate was filtered off, and the filtrate was concentrated to dryness on the water-bath. The residue was taken up by ether, the extract filtered and the ether removed. The clear, pale yellow, oily residue (0·75 g.) no longer gave the Liebermann-Burchardt reaction for cholesterol. By this treatment with digitonin the rat-unit was reduced from 80 mg. to 25 mg. The above yield therefore represents approximately 30 rat-units, showing that there is little loss in this method of purification.

Method of testing extracts.

This depends on the work of Stockard and Papanicolaou [1917] with the guinea-pig, which was extended to the rat by Long and Evans [1922]. It was demonstrated that a definite oestrous cycle exists in these animals, and that the various stages of the cycle can be accurately determined by examining smears from the vagina. Four stages of the oestrous cycle in the rat can be recognised: pro-oestrus, oestrus, post-oestrus and post-ovulation. The total duration of the cycle is 96 hours. The changes found in the smears depend on proliferation of the vaginal epithelium and keratinisation of these cells, proliferation of the uterine mucous membrane and migration of leucocytes. In cases of doubt, the animal may be killed and the genital organs examined both with the naked eye and microscopically.

Ovarian extracts were standardised by the method of Allen and Doisy, *i.e.* by determining the minimum amount which will bring about artificial oestrus in ovariectomised adult animals. Rats were used almost exclusively. The extracts were injected subcutaneously, dissolved in olive oil or suspended in weak bicarbonate solution. If the extract was active, oestrus appeared sometimes after 24 hours and usually within 36 hours of the time of injection.

The extracts containing much cholesterol were not easily absorbed and large "granulomata" appeared at the site of injection. When extracts freed from cholesterol were employed, practically no nodules resulted or they were much less marked. The total dose of extract to be given can be administered at one injection as absorption is quite slow. The same results were obtained from one dose as from a spread-out series of small doses which amounted to the same total. The responsiveness of the animals was found to wear off with the lapse of time after ovariectomy and larger doses became necessary for the production of oestrus. Thus 12 mg. of a liquor folliculi extract produced definite oestrus in a freshly spayed rat, while 50 mg. produced only slight pro-oestrus in a rat spayed 3 months previously. It must be remembered that 3 months corresponds to 30 oestrous cycles in the rat, which is equivalent to 2½ years in the human female. After 3 months the genital organs of ovariectomised rats are very thin and resemble fibrous cords. The effect of the preparation was also tested upon a female monkey: 100 mg. were injected subcutaneously, and within 3 days typical menstruation commenced. That this response was not the onset of a normal period was excluded by previous observations upon the animal. In a series of experiments in which the extract was administered by the mouth to ovariectomised rats, oestrus could not be induced even when very large doses were given.

Depressor effect.

Intravenous injection of active ovarian extract dissolved in olive oil was carried out on anaesthetised animals. As controls, solutions of cholesterol in olive oil were employed. As will be seen from Fig. 1 A the control injections produced a slight irregularity of the blood pressure in doses from 1–4 cc. The ovarian extract had a marked depressor effect; a fall of 24 mm. Hg resulting from injection of 0·1 cc. (Fig. 1 B) and a fall of 46 mm. Hg from a dose of 0·2 cc. (Fig. 1 C). With a dose of 1 cc., the depressor effect was more prolonged (Fig. 1 D). No marked effect on respiration was noted.

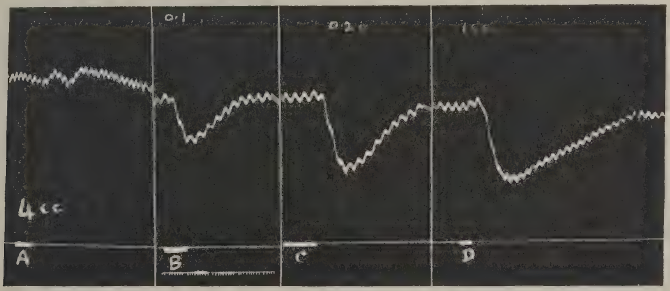

Fig. 1. Cat. Ether. Blood pressure. At A, 4 cc. of emulsion of cholesterol in olive oil injected. At B, ovarian extract in olive oil 0·1 cc. At C 0·2 cc. At D 1 cc. Time in seconds.

One experiment was carried out in which daily injections of ovarian extract were administered to a rat over a period of 14 days. One rat-unit was contained in each 1 cc. of the solution used and the injections were given daily in doses of 1 cc.

Smears from the vagina were studied. Oestrus appeared normally after 36 hours. Typical cornified cells were present till the sixth day of the experiment. No leucocytes or fibrin were found. On the sixth day a characteristic pro-oestrus smear appeared followed by oestrus cells on the seventh day.

Further pro-oestrus smears were obtained on the tenth day and succeeded by oestrus cells. On the fourteenth day the animal was killed. The uterine horns and body were distended and considerably congested. The vagina was widely dilated, *i.e.* typical oestrus conditions were present. Apparently the effect of continuous injection of the ovarian principle was greatly to prolong the period of oestrus. The post-ovulatory stage (appearance of leucocytes) was not noted and there was no resting stage; but oestrus lasting as long as 5 days was followed by a new pro-oestrus and then further oestrus. Throughout the period of the experiment the vaginal fluid had the characteristic watery appearance with small opaque granules like small rice grains as is seen in natural oestrus.

Interaction between insulin and the active principle.

That the onset of menstruation disturbs the carbohydrate metabolism to some extent has been pointed out by many workers.

Thus it is known that the advent of the period in a diabetic patient is often accompanied by an increase in the blood and urine sugar content, although the diet remains constant.

This suggested that there might be some interaction between insulin and the active principle of the ovary. Since the oestrous cycle has been fully worked out in mice, and their response to insulin is also well known, it was decided to employ these animals to test the point.

In the following tables can be seen the effect of varying doses of insulin upon mice not in oestrus, mice in natural oestrus, and mice in which artificial oestrus has been induced by injection of the ovarian principle.

A similar experiment was performed upon rabbits, the results of which appear in Table V.

Effect of insulin on female mice.

The mice were maintained at a temperature of 28°.

Table II. *Normal mice, not in oestrus.*

Insulin solution: a clinical solution diluted 1 in 300.

Dose cc.	Result
0·5	Completely comatose 1 hr. 25 mins. after injection. Revived by glucose
0·4	Comatose 2 hrs. after injection. Revived by glucose
0·3	Slightly sleepy condition 2 hrs. after injection
0·2	Nil
0·1	Nil

Table III. *Normal mice in natural oestrus.*

The same solution of insulin was injected as in Table II.

Dose cc.	Result
0·5 0·4 0·2 0·1	No effect visible up to 5 hrs later, when the mice were fed

Table IV. *Mice in artificial oestrus induced by injection of ovarian extract (from pig's ovaries).*

Insulin solution was a 1 in 100 dilution of a clinical solution.

Dose cc.	Mice in artificial oestrus	Non-oestrous controls
0·4	No apparent action	Comatose in under 2 hrs. and recovered on administering glucose
0·2		

Table V. *Effect of ovarian extract on the reaction to insulin of normal rabbits.*

Rabbit no.	Insulin weight mg.	Blood-sugar		
		Resting	1 hour	2 hours
Before injection of ovarian extract:				
9	0·5	110	70	40
12	1·0	98	40	29
14	1·5	120	33	19
After five injections 0·1 g. of ovarian extract at six hourly intervals:				
9	0·5	110	97	89
12	1·0	125	79	54
14	1·5	105	50	41

These experiments demonstrate the fact that the active principle of the ovary possesses the property of inhibiting the action of insulin. It is interesting to note that animals in natural oestrus respond in the same manner as those in which artificial oestrus has been induced.

SUMMARY.

1. A physiologically active principle can always be extracted from the ovary by means of alcohol, as a number of earlier workers have found.
2. The substance is an oil, soluble in alcohol, ether and acetone, and can be obtained free from cholesterol. Experiments are described showing its behaviour towards sodium ethoxide on saponification.
3. The method of standardisation is discussed, together with the general physiological properties.
4. By means of a series of injections an ovariectomised rat was kept in a state of continuous oestrus for a period of 14 days.
5. Administration by the mouth proved to be ineffective.
6. The extract was found to have a marked depressor action.
7. It was found that the substance inhibited the blood-sugar reducing action of insulin.

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CXXX. FACTORS INFLUENCING THE ACTION OF PANCREATIC LIPASE.

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In the course of a determination of the action of pancreatic lipase on certain esters, it was necessary to find a convenient method of preparing the enzyme in an active and pure condition, and to investigate the influence of certain factors with a view to obtaining the optimal conditions of action.

Method of Preparation.

The methods which have been devised for utilising the enzyme in the organ may be divided into three main groups: (*a*) the whole pancreas may be used, or a simple press juice obtained from it, (*b*) the enzyme may be extracted from the fresh gland with suitable solvents and the extract used in the crude state or subjected to further purification, (*c*) the pancreas may be treated so as to obtain a solid preparation containing the lipase. The solid obtained by this last method can be worked up to yield an enzyme in a high degree of purity as in the method of Willstätter and Waldschmidt-Leitz [1923].

Most of the previous work on the action of pancreatic lipase has been carried out with an enzyme preparation containing the natural activating substances. A determination of the effect of added substances on the activity of such a preparation is obviously of little value. Accordingly, we employed a method suggested by Rosenheim [1910] and developed by Umeda [1915]. The powder obtained by this method is claimed to be free from water-soluble substances, including enzymes, and organic substances soluble in alcohol, acetone and ether. It is found that practically all the amylase originally present in the extract is removed in the preparation of the powder, which however has still considerable tryptic activity. A sample of dried pancreas powder has been prepared by the method recommended by Willstätter and Waldschmidt-Leitz [1923], and the enzyme content of the two preparations compared under identical conditions. Lipase is present to the same extent in both samples, whilst in the preparation obtained by Willstätter's method, amylase is present, and the trypsin content is greater than in the powder prepared by Umeda's method.

In agreement with Virtanen [1924], we have found that the amount of lipase extracted by glycerol varies with the time allowed for extraction.

When, however, the gland is reduced by further mincing to a fine pulp, a few hours suffice for complete extraction of the enzyme. Willstätter and Waldschmidt-Leitz [1923] object to the use of alcohol in obtaining a solid preparation of the pancreatic enzymes. They find that treatment with alcohol causes an appreciable diminution in the activity of the lipase. We find that alcohol has no influence on the lipase in the powder obtained by Umeda's method, probably because this substance has already been washed with alcohol. We would suggest that the loss in activity noticed by Willstätter is due to the removal of an activating substance soluble in alcohol. In fact, Umeda has found that the so-called "co-enzyme" of lipase is made up of two fractions, one of which is soluble in alcohol. It is noteworthy, too, that Foster and Woodrow [1924] obtain a stable mixture of pancreatic enzymes by desiccating the organ with alcohol. Also, Vollmer, Schmidt and Serebrijski [1924], from the results of experiments on the antilipolytic effect of solutions of iodine in alcohol, state that the inhibition of these solutions is not due to the alcohol.

Estimation of Activity.

The activity of the lipase obtained from the pancreas has been determined in the following experiments by estimating the amount of acid liberated from ethyl butyrate or from olive oil. When the latter substance was used as the substrate, the technique developed by Willstätter and his collaborators was adopted. No antiseptics were added to the digestion mixtures, as Umeda [1915] and Long, Hull and Atkinson [1917] found this precaution unnecessary in the case of olive oil, and we have found in our work that ethyl butyrate has a pronounced inhibitory influence on bacterial growth.

A number of workers have directed attention to the inconsistent results obtained when a series of digestion mixtures are not all shaken to the same extent [cf. Bradley, 1909; Pekelharing, 1912; Haley and Lyman, 1921; and Willstätter, Waldschmidt-Leitz and Memmen, 1923]. We have used a device similar to that described by Armstrong and Gosney [1913], viz. shaking the test mixtures while in the incubator. The shaking would appear to be essential in this case to eliminate any discrepancies which might arise due to such physical causes as diffusion. Continued shaking was found to have no deleterious effect on the action of lipase on ethyl butyrate. On the contrary, a series of mixtures shaken for different periods up to 3 hours, were (at the end of 3 hours) hydrolysed to a degree proportionate to the time of shaking.

In the experiments on the activity of lipase at different hydrogen ion concentrations, we found that the amount of action could be easily and accurately determined by titrating a sample of the digestion mixture to the initial p_H with CO_2-free alkali, using a suitable indicator. Due regard was given to the concentration of indicator and to compensation for opalescence and colour of test solutions.

INFLUENCE OF HYDROGEN ION CONCENTRATION.

Little importance can be attached to the results of earlier work on the action of lipase owing to the failure to appreciate the necessity for regulating the reaction of the digestion mixture. Especially is this the case for the work on the influence of added substances. As Willstätter and his co-workers have shown in recent papers, a substance may have quite different effects according as the medium is acid or alkaline.

Under certain conditions, enzymes are considered to display maximal activity at a definite hydrogen ion concentration, which is termed the "optimum p_H." This property is regarded by many as characteristic of the particular enzyme. In seeking for the conditions for maximal activity of lipase it is necessary to determine and maintain this optimum p_H. Peculiar difficulties arise in the course of lipolysis due to the liberated acid changing the p_H of the medium. After various attempts had been made to eliminate the effect of the acid formed, we found that it was not possible to keep the p_H constant; but by using buffer solutions of suitable concentrations, the amount of change could be restricted. By increasing the concentration of the buffer solution to a point consistent with the accurate determination of the end point by titration, the range of p_H over which the enzyme was allowed to work could be reduced to as little as 0·2.

There are a number of values given in the literature for the optimum p_H of pancreatic lipase. Rona [1911] determined the action of lipase in different organ extracts at p_H 7·5, in phosphate buffer mixtures. Davidsohn [1912] found a reaction optimum of p_H 8·0 and in the following year [1913] he gave the value p_H 8·5. Further work of Rona and Bien [1914] suggests that there is an optimal zone over which the lipase of a pancreatic extract is most active, namely, from p_H 8·4 to p_H 9·0. Umeda [1915] gives a value of about p_H 7·4 for the action of the lipase in his extract of pancreas on olive oil in phosphate buffer solutions. With a saline extract of pancreas or with a pancreatic fistula secretion, Rona and Pavlovic [1923] obtained for the optimum p_H a value of 6·95 to 8·0. Some of the results, when represented by curves obtained by plotting activity against increasing p_H, do not show an optimal point or even a zone of maximal activity, but simply that a limiting value is being approached. The recent values obtained by Rona and Pavlovic, however, definitely prove that at p_H 3·7 there is no lipase activity and that above p_H 8·0 there is a distinct falling off in activity. Willstätter prefers to work with a buffer solution containing ammonia and ammonium chloride at a p_H of 9·2 at 18° when using olive oil as substrate, and a similar buffer solution at p_H 8·6 when tributyrin is the substrate. Anrep, Lush and Palmer [1925] carry out their lipase estimations in a phosphate buffer solution at p_H 7·8 and allow the reaction to proceed until the p_H is not less than 7·0.

Determination of optimum p_H.

The results of typical experiments carried out to determine the optimum p_H for pancreatic lipase are given in the curves in Fig. 1. Mixtures were made up as indicated in 50 cc. flasks of Jena glass fitted with rubber stoppers. All the solutions and vessels were previously warmed up to the reaction temperature. In all cases the substrate was added last to the digestion mixture. These were shaken mechanically in an incubator kept at 37°. To stop the reaction the flasks were plunged into ice-water for 10 minutes before titration. The results given are the mean of duplicate experiments, corrected for suitable controls, and are expressed in cc. CO_2-free alkali required to restore the initial p_H estimated colorimetrically and at room temperature. The buffer mixtures used are given by Clark [1922], Kolthoff [1925] and Palitzsch [1915].

Exp. 1. Mixture: 24·0 cc. KH_2PO_4—NaOH buffer solution (Clark).
0·5 cc. ethyl butyrate.
1·0 cc. suspension of 5 mg. of enzyme in water.

Incubated 3 hours at 37°, then titrated with $N/20$ NaOH using either bromothymol blue, phenol red or thymol blue as indicator.

The titration values are for 10 cc. of the reaction mixture (see curve, Fig. 1).

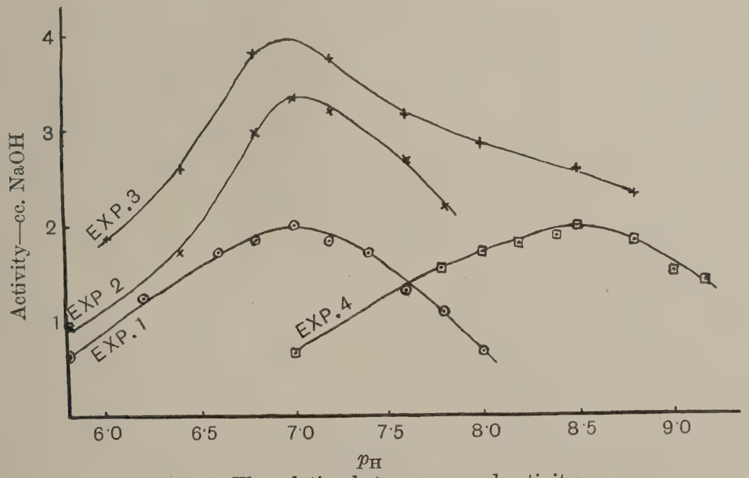

Fig. 1. The relation between p_H and activity.

Exp. 2. Mixture: 10·0 cc. phosphate buffer solution (Clark).
0·5 cc. ethyl butyrate.
1·0 cc. suspension containing 5 mg. enzyme.

Alkali used: $N/10$ NaOH.

Other conditions as in previous experiment (see curve, Fig. 1).

Exp. 3. Conditions as in previous experiment, but 10 cc. of Kolthoff's borax-phosphate buffer used instead of the phosphate solution above (see curve, Fig. 1).

Exp. 4. Conditions as in previous experiments.

Mixture: 46 cc. borax-boric acid-NaCl buffer solution (Palitzsch).
1 cc. ethyl butyrate.
2 cc. suspension containing 10 mg. enzyme.

The results are shown in the curve, Fig. 1.

It will be seen from these results that the optimal activity of pancreatic lipase in phosphate and phosphate-borax buffer solutions with ethyl butyrate as substrate occurs at about p_H 7·0, whilst in Palitzsch's buffer mixture there is a zone of maximal activity about p_H 8·5.

The following experiments were carried out with a crude glycerol extract of pig's pancreas. 0·5 cc. of the extract, which had been strained twice through muslin, was diluted with 5 cc. of phosphate buffer solutions of various hydrogen ion concentrations, and 3 cc. of water, 0·5 cc. of ethyl butyrate and 0·5 cc. of toluene were added. After 3 hours' shaking at 37° the mixture was titrated to a pink colour with $N/10$ NaOH, using phenolphthalein as indicator.

Exp. 5.

p_H	Mean titration value (corr.) cc.
5·8	3·53
6·2	3·94
6·8	4·86
7·0	5·20
7·4	5·35
7·6	5·51
7·8	6·55

The experiment was repeated with the same quantities of extract, ester and toluene in 25 cc. of the buffer mixtures. 10 cc. portions were titrated as in the preceding experiment after 3 hours' incubation.

Exp. 6.

p_H	Mean titration value (corr.) cc.
6·2	3·4
7·0	5·6
7·4	5·6
7·6	5·75
7·8	6·1
8·0	6·25

From a consideration of the foregoing results, it is easy to understand why anomalies occur in the findings of different workers. The substances accompanying the lipase in the crude glycerol extract appear to protect the enzyme from destruction in the more alkaline media.

The effect on lipase action of a preliminary treatment with buffer solutions of different p_H.

The optimum p_H of pancreatic lipase may be considered as the outcome of two opposing tendencies. With increase in alkalinity an increase in hydrolytic activity takes place, but at the same time, the enzyme is less stable.

This idea is suggested by the work of Noyes, Suguira and Falk [1923], and is borne out by the results of the following experiments.

10 mg. of enzyme were added to 15 cc. of phosphate buffer solution (Clark) of various p_H. This suspension was shaken in the incubator for 24 hours at 37°. Each sample was then adjusted with the calculated amounts of alkali, phosphate solution or water, so that the final p_H was 7·6 and the salt concentration of the mixtures was the same in all cases. 0·5 cc. of ethyl butyrate was added and the mixture shaken again in the incubator for 24 hours, when 10 cc. aliquots were titrated with $N/10$ NaOH to p_H 7·6, using phenol red as indicator.

Exp. 7.

p_H	Mean titration value (corr.) cc.
6·6	3·35
7·0	3·25
7·2	3·29
7·4	3·33
7·6	2·88
7·8	2·35
8·0	1·90

A similar experiment was carried out using Clark's borax-boric acid-KCl buffer mixtures. All the mixtures, after 24 hours' contact with the respective solutions, were adjusted to p_H 8·6, and the activity of the enzyme estimated by its action on ethyl butyrate for 24 hours.

Exp. 8.

p_H	Mean titration value (corr.) cc.
7·8	1·92
8·0	2·19
8·2	1·66
8·6	1·42
9·2	1·40

A study of the relationship between p_H and lipase activity in the various buffer solutions, and of the effect of a preliminary treatment with buffer solutions of different p_H before estimating the activity, suggests that the "buffer value" of the mixtures determine to some extent the form of the optimum curves. The position of the optimal zone when Palitzsch's solutions are used coincides with the position of the maximum buffer value. Also, when the buffer value of the phosphate solutions is increased on the alkaline side by the addition of borax, as in the Kolthoff buffer mixtures, an increased activity is observed.

THE EFFECT OF ADDED SUBSTANCES ON THE ACTION OF PANCREATIC LIPASE.

Substances added to lipase may affect its action by establishing a favourable p_H, by maintaining this p_H because of buffer value, or by some specific influence, probably chemical in nature, on the enzyme. In an experiment in which lipase was introduced into a solution of ethyl butyrate at a p_H suitable for lipolytic action, no lipase action could be detected by comparing the rate of change of p_H of this mixture with that of a similar solution of ethyl butyrate containing no enzyme.

The enzyme for this experiment was obtained from the powder prepared by Umeda's method, and was freed as far as possible from accompanying substances by digestion in 90 % glycerol and subsequent filtration through a hardened filter paper, with suction. A clear solution was thus obtained containing a considerable amount of lipase. This was added to a solution of pure ethyl butyrate in CO_2-free distilled water. A control experiment was set up in which the same amounts of ester solution and glycerol were present. Samples were withdrawn at frequent intervals and the p_H estimated colorimetrically. Special precautions were taken to prevent contamination from the vessels used and from CO_2 in the atmosphere. It was found that the time taken for the change in p_H from 7·8 to 7·05, using the same indicator, phenol red, was identical in both experiments. Apparently some activating substance must be added to the enzyme before it can function even at a suitable hydrogen ion concentration.

The influence of phosphates.

In determining the most suitable concentration of buffer solution required to restrict the amount of change in p_H during the course of lipolysis, it became necessary to investigate the effect of various concentrations of phosphate solutions on lipase activity at a definite initial p_H. The following are typical of the results obtained.

A series of mixtures was prepared containing 20 mg. of enzyme in 10 cc. of phosphate buffer solution and 0·1 cc. of ethyl butyrate. Various salt concentrations were used, the initial p_H being 7·6. After shaking for 1 hour in the incubator, the mixtures were titrated with $N/20$ NaOH to p_H 7·6, using phenol red as indicator.

Exp. 9.

Molar concentration of phosphate	Mean titration value (corr.) cc.
—	0·44
0·005	0·99
0·01	1·2
0·02	1·48
0·025	1·57
0·03	1·82
0·04	2·00
0·05	2·23
0·075	2·08

The range of concentration was increased in the following experiments. Buffer mixtures at p_H 7·6 and 7·2 were made up, the concentrations of phosphate being 0·2M. These solutions were diluted to give solutions of the required salt content. The mixtures contained 25 cc. phosphate solution, 0·5 cc. ethyl butyrate and 1 cc. suspensions containing 10 mg. of enzyme.

10 cc. aliquots were titrated at the end of 3 hours' incubation with $N/20$ NaOH, using phenol red as indicator for the first and bromothymol blue for the second series. 10 cc. portions were titrated again after the reaction had proceeded for a further 21 hours.

Exp. 10. The results are shown in the curves of Fig. 2.

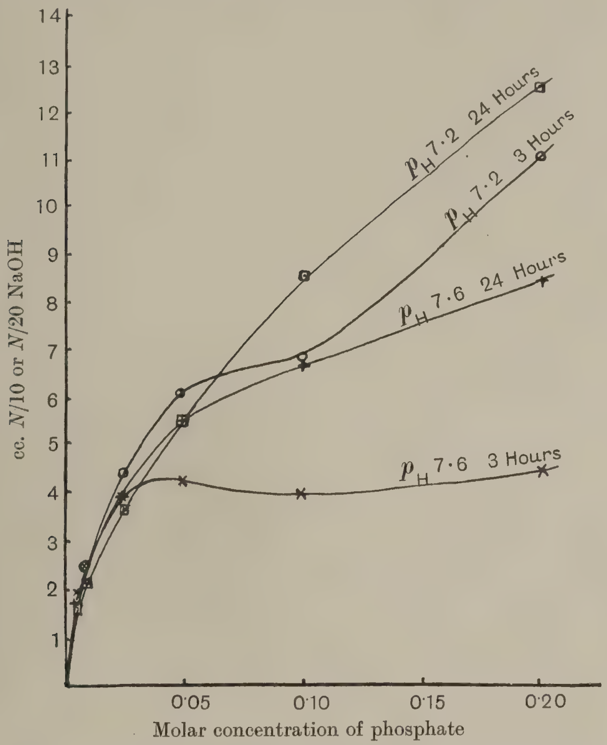

Fig. 2. The influence of phosphate concentration (see Exp. 10).

The experiment was repeated using different phosphate concentrations.

Exp. 11. p_H 7·6.

Concentration of phosphate M	Mean titration value (corr.) 3 hours cc.	24 hours cc.
0·025	1·77	5·07
0·05	1·91	7·36
0·075	1·68	7·32
0·1	1·63	7·96
0·15	1·72	8·72
0·2	1·75	9·62

It will be seen from these results that Clark's phosphate buffer solutions of the usual concentration ($0·05M$ KH_2PO_4) are suitable media for carrying out lipase tests on ethyl butyrate as substrate.

A straight line relationship between the concentration of the phosphate solution and the activity of the lipase would be anticipated if only a buffer effect were involved. The relationship is no doubt modified by a specific salt effect similar to that which is found in the case of synthetic bile salts. When the amount of lipase action is increased by carrying out the reaction at a p_H nearer the optimum value, or by lengthening the reaction period, a closer approximation to this relationship is observed.

Solutions of sodium α- and β-glycerophosphates, when compared with a solution of potassium phosphate of the same molar concentration and at the same initial p_H, were found to activate lipase to a greater extent, when tested under the same conditions. The two glycerophosphates were identical as regards their activating powers, thus suggesting that the phosphate ion is responsible for the activation. The reason for the different effects of these salts probably lies in their buffer values, which is found to be greater for the glycerophosphates than the inorganic phosphate at the p_H employed. In view of the above results, the objection raised by Noyes, Suguira and Falk [1923] to the use of buffer solutions in the estimation of lipase cannot be sustained.

The adjuvant action of phosphates has been clearly demonstrated by Umeda [1915]. Also, Long, Hull and Atkinson [1915] adduced evidence that the salts present in the pancreas are mainly phosphates. One function of these phosphates is evidently to activate the lipase of the pancreatic juice. Willstätter, Waldschmidt-Leitz and Memmen [1923], by adding calcium chloride, albumin and an ammonia-ammonium chloride buffer mixture, claim to have produced a medium in which lipase from any source is completely activated. We find that the addition of small amounts of phosphate increases the action of pancreatic lipase, even when the system of activators recommended by Willstätter is present. Thus, in one of a number of similar experiments on the effect of small amounts of phosphate solution on the action of lipase on olive oil, we find, by using Willstätter's technique, that 4·05 cc. 0·3M alcoholic KOH are required to neutralise the acid formed from 2·5 g. olive oil. Increased amounts of the same alkali are required, 5·8, 4·8 and 4·7 cc., when 0·25, 0·5 and 1·0 cc. respectively of the elution mixture, used in the process of the purification of lipase described by Willstätter and Waldschmidt-Leitz [1923], are also present. The above titration figures are the mean of the results of duplicate experiments, corrected for the necessary control. The enzyme powder used in this experiment was prepared by Willstätter's method and was found to have an activity of the same order as that given in this author's paper. When in the later stages of purification of lipase Willstätter tested for the amount of enzyme, the estimations were carried out in the presence of amounts of elution mixture similar to those used above. The lipase values would therefore not be comparable with the initial values obtained in mixtures not containing phosphate.

The influence of bile salts.

It is remarkable that the results of a number of workers, de Jonge [1917], Orbeli and Tetyaeff [1916] and Terroine [1920], on the influence of various concentrations of bile salts on the activity of pancreatic lipase show the same relationship as we have observed for the effect of increasing amounts of phosphates. Anrep, Lush and Palmer [1925] have attributed to bile salts a specific chemical influence, distinct from their surface tension properties, which they hold in common with substances such as caprylic alcohol and saponin. We would suggest that the specific chemical effect, which both phosphate and bile salts can exert, is partly responsible for the connection observed between salt concentration and enzyme activity. It is probable also that the same factor causes the shift in optimum temperature of lipase in the presence of bile salts. Terroine [1920, p. 199] gives figures which show that an elevation of the optimum temperature of lipase in pancreatic juice occurs when bile salts are present, but he does not comment on this fact. In some preliminary work on this point, we have obtained results similar to those of Terroine.

The effect of albumin and edestin.

Emphasis is laid by Falk [1921] on the protein structure of lipase. The preparations we have obtained at different times contain amounts of nitrogen ranging from 8·5–12·3 %. Moreover, the activity of a definite amount of each sample is directly proportional to the amount of nitrogen in the powder. We do not take these results as evidence of the protein nature of the enzyme, but rather that the enzyme is associated with a protein substance and has been freed from impurities to varying extents, depending upon the efficiency of the washing. The accompanying protein, however, largely determines the properties of the enzyme. Falk [1915] has stated that he has been able to separate the ester-hydrolysing enzymes of castor bean into a "lipase" and an "esterase," the former being the globulin fraction, the latter an albumin-like substance. The following experiments were performed to show that the enzyme is not necessarily a protein, but it can manifest so-called "esterase" or "lipase" properties according as the same enzyme material is accompanied by one or other of the two proteins.

A clear, filtered glycerol extract of dried pancreas was used as the source of lipase. The albumin was crystalline egg albumin (Boots), and the globulin was edestin. The mixtures prepared were:

(1) 18 cc. distilled water.
0·5 cc. glycerol extract of enzyme in 2 cc. of water.
0·5 cc. ethyl butyrate.

(2) 14 cc. distilled water.
4 cc. solution containing 10 mg. albumin.
0·5 cc. glycerol extract in 2 cc. water.
0·5 cc. ethyl butyrate.

(3) As (2) but 20 mg. albumin.

(4) As (2) but 10 mg. edestin.

(5) As (2) but 20 mg. edestin.

(6), (7), (8), (9) and (10) are comparable with the above, except that 2·5 g. of olive oil are used as substrate instead of ethyl butyrate. The results are expressed in terms of $N/10$ NaOH required to neutralise the butyric acid liberated in the first series after 18 hours' shaking in the incubator at 37°. In the experiments with olive oil, the titration was carried out in alcohol-ether solution with $0{\cdot}397N$ alcoholic KOH. Phenolphthalein was used as indicator in both cases. The values recorded are the mean of duplicate determinations corrected for the necessary control. The results of two experiments, using different glycerol extracts, are given in the following table.

Exp. 12.

		Substrate				
		Ethyl butyrate			Olive oil	
Mixture.		Exp. 1	Exp. 2		Exp. 1	Exp. 2
Enzyme and substrate		cc.	cc.		cc.	cc.
Plus water	(1)	0·43	0·48	(6)	1·6	0·99
Plus 10 mg. albumin	(2)	1·9	1·2	(7)	0·45	0·57
Plus 20 mg. albumin	(3)	2·5	1·85	(8)	0·49	0·83
Plus 10 mg. edestin	(4)	1·15	0·75	(9)	1·8	1·33
Plus 20 mg. edestin	(5)	1·4	1·0	(10)	1·8	1·90

Clearly from these results, the same enzyme, associated with albumin, hydrolyses a simple ester such as ethyl butyrate more easily than a typical glyceride, olive oil, which however is decomposed to a greater extent than ethyl butyrate when the lipase is allowed to work in association with edestin. It seems that Falk's conclusions were based on the isolation of the two different proteins, on which the same enzyme was adsorbed, rather than a separation of two ester-splitting enzymes.

The action of trypsin on lipase.

Conflicting evidence is found in the literature on this subject. We find that Lewkovitsch and Macleod [1903], Mellanby and Woolley [1914], Terroine [1920] and Mellanby [1925] state that trypsin destroys lipase. On the other hand, Shaw-Mackenzie [1915] and de Souza [1916] find that lipase and trypsin may be preserved together in extracts of pancreas containing more than 30 % glycerol. The glycerol is supposed to inhibit the action of trypsin on lipase, and on dilution the protective action of the glycerol is removed. We have found, however, that in solutions containing up to 30 % glycerol it is possible for trypsin to decompose caseinogen. Further, if trypsin is allowed to act in solutions containing active lipase, the loss of lipase is not so marked if due regard is given to the destruction of protein activating substances of the lipase. Willstätter and Memmen [1924] have remarked on the instability of lipase in aqueous extracts of pancreas. They find that the lipase is destroyed even when all the trypsin is removed from solution.

The following experiments were conducted to determine the nature and extent of the supposed destructive action of trypsin on lipase. Solutions of lipase were subjected to the action of trypsin. The amount of lipase in these mixtures was estimated after intervals by the action on olive oil, in the presence of protein activators, or on ethyl butyrate.

Exp. 13. A mixture (A) was prepared containing:
5 cc. phosphate buffer solution at p_H 7·8.
200 mg. pancreas powder (Willstätter).
20 cc. neutral "liquor pancreaticus" (Benger).
25 cc. water.

A second mixture (B) was prepared in which the "liquor pancreaticus" had been boiled before addition.

These mixtures were maintained at a temperature of 37°. All the solutions and vessels used were previously warmed up to this temperature. To estimate the amount of lipase, 4 cc. of each mixture were withdrawn and added to 10 cc. of phosphate buffer solution at p_H 7·8, 0·5 cc. of ethyl butyrate then being added. The estimations were carried out in duplicate with the necessary controls. After incubating for 1 hour, the amount of $N/10$ NaOH required for neutralisation of the acid formed was determined in the usual manner.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	6·65	5·85
1	4·25	5·6
3	1·55	2·75

Exp. 14. The following results were obtained from a similar experiment, using the mixture (A):
100 cc. phosphate buffer at p_H 8·2.
200 mg. pancreas powder.
30 cc. neutral "liquor pancreaticus."

As before, mixture (B) contained boiled "liquor pancreaticus." For the lipase test, 10 cc. of the mixture were withdrawn, 0·5 cc. of ethyl butyrate added and the estimation carried out as above.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	5·8	5·95
1	2·2	4·45
2·5	0·8	2·5
4·5	0·5	1·18

Willstätter's technique for the estimation of the amount of lipase was used in Exps. 15 and 16. For each test, 2 cc. of mixture were withdrawn. 0·398 N alcoholic KOH was used for titration of the acid liberated from the olive oil.

Exp. 15. Mixture (A) contained:
1·25 cc. phosphate buffer solution at p_H 7·8.
50 mg. pancreas powder.
5 cc. neutral "liquor pancreaticus."
6·25 cc. water.

In the case of mixture (B), the "liquor pancreaticus" had been boiled before addition. The following results were obtained:

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	2·6	3·15
3	1·1	1·65
24	0·22	0·23

Exp. 16. In this experiment, mixture (A) contained:

2·5 cc. phosphate buffer solution at p_H 7·8.
100 mg. pancreas powder.
20 cc. neutral "liquor pancreaticus."
2·5 cc. water.

Time of incubation before testing	Mean titration value (corr.)	
	Mixture A	Mixture B
hours	cc.	cc.
0	4·94	5·16
2	3·04	3·34
4	2·0	2·78

The amount of trypsin in the mixtures containing unboiled "liquor pancreaticus" was determined and was found to be 4–6 times as great as in the mixtures which contained boiled "liquor pancreaticus."

Experiment showed that there is no measurable tryptic decomposition of the small amounts of albumin present when Willstätter's technique is used.

From the results of the preceding experiments it is seen that there is an appreciable difference in the lipase contents of the various mixtures if no allowance is made in the estimations for the loss by tryptic action of protein activating substances of the lipase. When large amounts of trypsin are present and the lipase estimations are carried out by the ethyl butyrate method, the diminution in lipase content is very pronounced (Exps. 13 and 14). If, however, albumin is added to compensate for protein losses by tryptic action, as in the tests by Willstätter's method, the rate of loss of lipase is not very different from that of the solutions to which no further amounts of trypsin had been added (Exps. 15 and 16).

The effect of formaldehyde.

De Souza [1916] has found that formaldehyde in concentrations of 1 : 1000–1 : 500 does not destroy lipase. Palmer [1922] has shown that solutions containing 0·1–0·05 % of formaldehyde have no effect on lipase action. These results are important in view of the theory of the protein nature of lipase. We have obtained the following results which are in agreement with the foregoing.

Mixtures were made up containing:

20 cc. phosphate buffer solution at p_H 7·2.
5 cc. formaldehyde solution.
5 mg. enzyme powder in 1 cc. buffer solution.
0·5 cc. ethyl butyrate.

Test mixtures, in duplicate, with necessary controls, were shaken in the incubator for 3 and 24 hours at 37°. The results are expressed in cc. of $N/10$ NaOH required to restore the p_H of 10 cc. to the initial value, using bromothymol blue as indicator.

Exp. 17.

Concentration of formaldehyde	Mean titration value (corr.) 3 hours cc.	Mean titration value (corr.) 24 hours cc.
0	7·25	10·75
1 : 2500	6·16	10·4
1 : 2000	6·06	10·4
1 : 1500	5·73	9·9
1 : 1000	5·82	9·8
1 : 500	5·32	8·4
1 : 100	2·64	6·3

An experiment carried out in the same manner gave the following results:

Exp. 18.

Concentration of formaldehyde	Mean titration value (corr.) 3 hours cc.	Mean titration value (corr.) 24 hours cc.
0	3·7	8·8
1 : 2000	3·02	7·1
1 : 1000	3·05	7·7
1 : 500	3·29	7·6
1 : 100	1·35	5·2
1 : 50	0·90	2·9

For concentrations of formaldehyde less than 0·2 %, especially for the longer reaction periods, the formaldehyde appears to have but little inhibitory influence. If the enzyme had the structure of a typical protein the effect of the formaldehyde would no doubt be much more pronounced than the results suggest.

SUMMARY AND CONCLUSIONS.

Lipase can be conveniently estimated by titration of the acid formed from ethyl butyrate, when the reaction is carried out in a phosphate buffer medium.

The optimum p_H for the action of pancreatic lipase is determined by the substances present in the reacting mixture. With a purified pancreas powder containing lipase, the optimum p_H in phosphate and in phosphate-borax buffer solutions was at p_H 7·0, and in borax-boric acid buffer solutions there was an optimal zone about p_H 8·4. The lipase of a glycerol extract of pig's pancreas in phosphate buffer mixtures, acting on ethyl butyrate, showed maximum activity at p_H 7·8, but gave no definite p_H optimum.

The enzyme does not promote the hydrolysis of ethyl butyrate in the complete absence of activators, even if the medium has favourable hydrogen ion concentration.

Increasing concentrations of phosphates and bile salts have a similar effect on the activity of lipase. In both cases, when the values of the activities are plotted against increasing salt concentration, a curve is obtained showing first, a rapid rise, then a plateau, followed by another rise. The form of the curve appears to be determined by both the buffer value of the solutions and a specific influence of a chemical nature on the enzyme.

Phosphates should be present in any system of activators intended for complete activation of pancreatic lipase.

Our results suggest that the protein structure which has been ascribed to lipase is characteristic of the substances associated with the enzyme rather than of the enzyme itself. The ester-hydrolysing properties of the enzyme have been definitely altered by changing the accompanying protein. The apparent destruction of lipase by trypsin is due, in part, to the removal by hydrolysis of proteins which activate the lipase.

Formaldehyde has no appreciable effect on pancreatic lipase action in concentrations less than 0·2 %.

We wish to acknowledge our indebtedness to Prof. Cohen, in whose laboratory and under whose guidance the investigation was carried out, to the Royal Society for a grant which covered part of the cost of the materials, and to the Department of Scientific and Industrial Research for grants which have enabled us to devote our whole time to the research.

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CXXXI. HYDROLYTIC DISSOCIATION CURVES.

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(*Received August 20th, 1925.*)

When watery solutions of a weak base, such as urea, and a strong acid, for example hydrochloric acid, are mixed together part of the base and part of the acid unite to form a salt, the rest of the base and acid remaining in the free state. This relation is represented by the equation

$$\frac{[\mathrm{U}]\times[\mathrm{HCl}]}{[\mathrm{U.HCl}]}=H, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1)$$

the quantities in brackets representing molecular concentration of base, acid and salt respectively and H being a constant[1]. A similar equation holds for a weak acid and a strong base.

Presumably some of the interactions met with in biochemical work, in which one substance is observed to have an inhibitory or restraining influence upon the action of another, are of the above type. The object of the present paper is to collect together some of the equations and curves which will be found of value in investigating such interactions.

The case may be considered in which two antagonistic substances of unknown molecular constitution are mixed in concentrations represented by d and m respectively. If the reaction taking place is similar to that occurring when watery solutions of a weak base (d) and a strong acid (m), or of a weak acid and a strong base, are mixed together, then the relation expressed above would, of course, apply here also. Since, however, the concentrations would in general be given in percentage form, the molecular concentration being unknown, it would usually be necessary to multiply the percentage concentration of one of the two antagonistic substances, for example d, by a dilution factor f, so chosen that df would represent the molecular concentration of d relative to that of m taken as a standard. If m is found after admixture to have been reduced to c then $m - c$ would represent the amount of salt formed and $df - (m - c)$ would give, in terms of this standard, the concentration of free substance corresponding to d, the equation for the relation in question being

$$\frac{c\,[df-(m-c)]}{m-c}=H. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(2)$$

In this equation there are three variables, c, d and m, and two constants H and f. Of the three variables d and m are known, being the respective concentrations added at the commencement of the experiment, whilst c has to be estimated by an appropriate method. From the values of c, d and m obtained in different experiments H and f are determined.

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[1] H is used instead of the symbol K in order to indicate that a special equilibrium dependent upon hydrolysis is being dealt with.

Since equation (2) contains three variables the individual experiments will be represented by points lying upon a curved surface and in general cannot be exhibited on a plane. If, however, the initial concentration of d or of m is maintained unchanged in all experiments, or if the relation of the two, $\frac{d}{m}$, is kept constant, then the variables become, in effect, reduced to two and the experiments will lie upon plane curves. Such curves may be conveniently referred to as d, m and A curves $\left(\text{where } A = \frac{df}{m}\right)$. Of these the last, which will alone be considered here, possess the advantage that the two substances may first be mixed in bulk with any desired degree of accuracy and then for each experiment a measured amount of the mixture taken: in this way the error introduced by making two separate measurements, one for each substance, is avoided.

For A curves equation (2) may be written

$$\frac{c\,[m(A-1)+c]}{m-c} = H. \quad \text{.............................}(3)$$

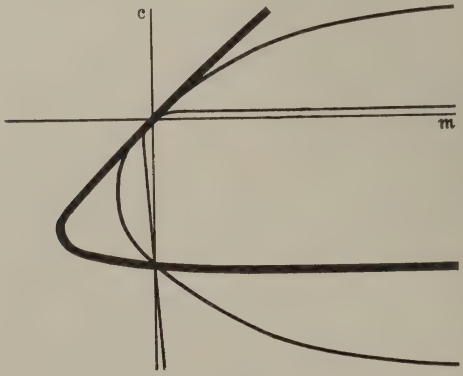

Fig. 1.

Curves of this type are shown in Fig. 1, the abscissae representing values of m and the ordinates corresponding values of c. The upper (thick) and lower (thin) curves are hyperbolic, corresponding to $A < 1$ and $A > 1$ respectively, whilst the middle curve, for which $A = 1$, is a parabola. The asymptotes of the hyperbolic curves, which are not shown in the figure but are nevertheless sufficiently obvious, are given by

$$c = \frac{H}{A-1} \text{ and } c = -(A-1)\,m - \frac{AH}{A-1}.$$

Only the upper portions of the curves, having positive values of c and m, are of importance in experimental work.

If the constants are known the values of c may be calculated from the equation

$$c = \frac{m - df - H}{2} \pm \sqrt{mH + \left(\frac{m - df - H}{2}\right)^2}, \quad \text{.................(4)}$$

which may also be written

$$c = \frac{m(1 - A) - H}{2} \pm \sqrt{mH + \left(\frac{m(1 - A) - H}{2}\right)^2}. \quad \text{.........(4 a)}$$

The determination of the constants may be attended with difficulty. If c, d and m are known with a considerable degree of accuracy H and f can be ascertained from any two experiments by the aid of the following equations:

$$H = \frac{c_1 c_2 [d_2 (m_1 - c_1) - d_1 (m_2 - c_2)]}{c_1 d_1 (m_2 - c_2) - c_2 d_2 (m_1 - c_1)}, \quad \text{.......................(5)}$$

$$f = \frac{(c_1 - c_2)(m_1 - c_1)(m_2 - c_2)}{c_1 d_1 (m_2 - c_2) - c_2 d_2 (m_1 - c_1)}. \quad \text{..........................(6)}$$

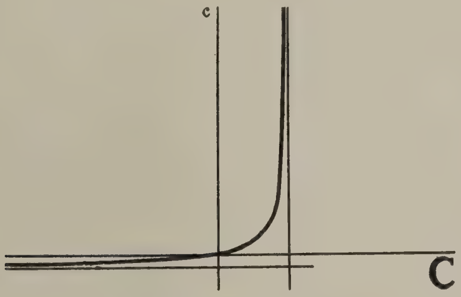

Fig. 2.

If, however, the estimation of c, d and m is not susceptible of a high degree of exactitude, the above formulae, which unfortunately are determined by relatively small differences existing between the variables, may fail even to indicate the order of magnitude of the constants. The search for suitable values then becomes both difficult and laborious. The method I have adopted when faced with this dilemma has been in the first place to determine the approximate limits of the ratio $\frac{H}{f}$ and then, choosing arbitrarily a series of values of H and f lying within these limits, to calculate by the aid of equation (4) c for the experiments made, eventually selecting such values for the constants as afford the best agreement between c-calculated and c-observed. The ratio $\frac{H}{f}$ is determined approximately by using for experiment increasingly high values of d until (1), even when large amounts of m are employed, c is small compared with m and (2) the ratio, calculated from the equation given below, does not vary markedly in experiments extending over the largest possible range. When the latter condition is fulfilled it may be concluded that df is large compared with $m - c$. We may therefore disregard

$m-c$ in the numerator of the expression on the left of equation (2). This equation then becomes with close approximation

$$\frac{cdf}{m-c}=H$$

or

$$\frac{H}{f}=\frac{cd}{m-c}. \qquad \text{.......................(7)}$$

In an attempt to determine the nature of the interaction taking place between two antagonistic substances the expression $\frac{m-c}{d}$ is of importance. If this expression is represented by C and introduced into equation (2) we obtain

$$c=\frac{C}{f}(H+c). \qquad \text{.......................(8)}$$

The curve (Fig. 2) corresponding to this equation is a hyperbola the asymptotes of which are given by $C=f$ and $c=-H$. If the interaction between d and m is a chemical combination $\frac{m-c}{d}$ is merely a mathematical expression having no special significance. If, however, the interaction is an adsorption process then $\frac{m-c}{d}$ indicates the concentration in d of the adsorbed fraction of m, d being in this case a hydrosol whose particles adsorb m. The equilibrial equation for an adsorption process is

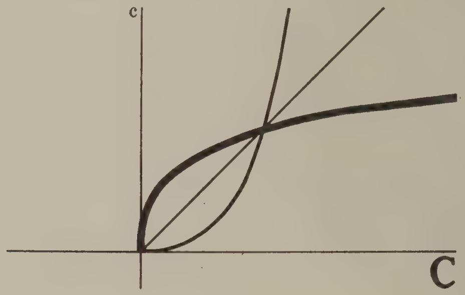

Fig. 3.

$$c=pC^q \qquad \text{.......................(9)}$$

where p and q are constants. The types of curve given by equation (9) are shown in Fig. 3 (for $p=1$): if $q>1$ a curve which is concave upwards is obtained, if $q<1$ the curve is concave downwards, while for $q=1$ a straight line is given. The first curve, shown thin in the figure, alone corresponds to an adsorption process and should be compared with that of equation (8) shown in Fig. 2. In any two experiments

$$\frac{c_1}{c_2}=\left(\frac{C_1}{C_2}\right)^q \qquad \text{.......................(10)}$$

Hence

$$\frac{\log\frac{c_1}{c_2}}{\log\frac{C_1}{C_2}}=q. \qquad \text{.......................(11)}$$

It is thus seen that in experiments to which equation (9) is applicable the ratio of the logarithms of observed values of $\frac{c_1}{c_2}$ and $\frac{C_1}{C_2}$ is constant.

Another curve of value is that obtained by plotting together values of c and H from

$$\frac{c\,[m\,(A-1)+c]}{m-c}=H, \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(3)$$

when m and A are constant. Three such curves are shown in Fig. 4, the top one corresponding to $A < 1$, the lowest to $A > 1$ and the middle to $A = 1$, the value of m being the same for all three: the curves are hyperbolic, the line $c = m$ parallel to the axis of abscissae, shown in the figure, being the asymptote of all curves having the same value of m. (The broken parabola, also shown in the figure, is the locus of vertical tangents to the curve: its equation is $H = -\frac{c^2}{m}$.) Under the conditions of experiment obtaining when the curve is nearly horizontal, as is the case with the upper curve, for example, a slight error in the estimation of c gives rise to a considerable error in the estimation of H. This is sometimes a source of embarrassment in experimental work when it is found that values of the constants varying over a wide range, but preserving the same value of the ratio $\frac{H}{f}$, nevertheless afford good agreement between calculated and observed values of c. In order that the value of H may be sharply defined it must lie upon a steep curve obtained by suitably adjusting the concentrations of m and d employed.

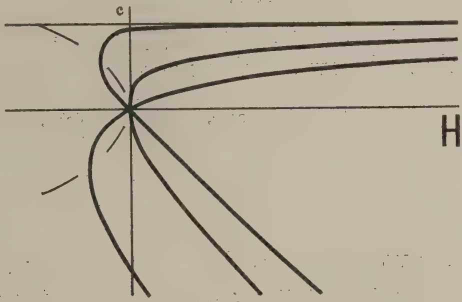

Fig. 4.

Before proceeding further it will be of interest to refer very briefly to the combination of a weak base with a weak acid. Here the equation of equilibrium is

$$\frac{[\text{base}]\times[\text{acid}]}{[\text{salt}]^2}=H. \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(12)$$

The investigation of the figures corresponding to this equation is similar to that for equation (1).

By way of illustration of some of the difficulties and limitations encountered the constants H and f will now be determined for the series of estimations of the amount of hydrochloric acid remaining free (c) in mixtures of urea (d) and (total) hydrochloric acid (m) given below [Walker, quoted by Abegg, 1903]. The quantities represent amounts in grams contained in 1 cc. of the mixture.

Exp.	d	m	c
1	0·03	0·0365	0·0275
2	0·06	,,	0·0213
3	0·12	,,	0·0131
4	0·18	,,	0·0095
5	0·24	,,	0·0069

The values of H and f calculated by the aid of equations (5) and (6) for individual pairs of experiments are as follows:

Exps.	H	f
1, 2	0·048	0·82
2, 3	0·019	0·47
3, 4	0·051	0·95
4, 5	0·013	0·35
5, 1	0·025	0·58
	Mean 0·0313	Mean 0·634

The range of values is large, especially so for H.

The values of $\frac{cd}{m-c}$ for the series (cp. equation (7)) are:

Exp.	$\frac{cd}{m-c}$
1	0·092
2	0·084
3	0·067
4	0·063
5	0·056

The ratio $\frac{H}{f}$ is therefore less than 0·056.

If c is calculated by means of equation (4) for $H = 0{\cdot}0313$ and $f = 0{\cdot}634$ the concentrations shown below are obtained:

Exp.	$c_{\text{calc.}}$	$c_{\text{obs.}}$
1	0·0276	0·0275
2	0·0212	0·0213
3	0·0135	0·0131
4	0·0096	0·0095
5	0·0074	0·0069

By diminishing the values of H and f closer agreement between $c_{\text{calc.}}$ and $c_{\text{obs.}}$ was reached. The values ultimately selected were $H = 0{\cdot}0285$ and $f = 0{\cdot}608$. With these the calculated concentrations of c were:

Exp.	$c_{\text{calc.}}$
1	0·0275
2	0·0210
3	0·0133
4	0·0094
5	0·0072

Since the molecular weights of hydrochloric acid and urea are known it is of course unnecessary to determine f, the theoretical value of which is $\frac{36{\cdot}5}{60} = 0{\cdot}6083$. The determination has been made above in order to illustrate the procedure followed when the molecular weights of the interacting substances are unknown. Walker's mean determination (in absolute measure) of H is 0·78 corresponding to $0{\cdot}78 \times 0{\cdot}0365 = 0{\cdot}0285$ when c, d and m are given in amounts per cc.

If it is desired to test the applicability of equation (9) q may be calculated by means of equation (11). The result is given below:

Exps.	q
1, 2	1·47
2, 3	1·84
3, 4	1·23
4, 5	1·62
Mean	1·54

Taking $q = 1{\cdot}54$, a mean value of $p = 0{\cdot}173$ is obtained. If c is calculated from the equation

$$c = 0{\cdot}173C^{1{\cdot}54}$$

for the values of C obtaining in Walker's experiments a close agreement with the observed amounts is seen:

Exp.	$c_{\text{calc.}}$	$c_{\text{obs.}}$
1	0·0270	0·0275
2	0·0209	0·0213
3	0·0140	0·0131
4	0·0093	0·0095
5	0·0069	0·0069

The unexpected result is thus obtained that equations (2) and (9) are equally applicable over the range of experiments made. No indication is therefore afforded as to whether hydrolytic dissociation, corresponding to the former equation, is involved or a process corresponding to the latter. When the curves given by equations (8) and (9) are constructed it is found that the two are nearly identical within the limits examined. Beyond these limits, however, a choice is possible, the curves separating and the divergence for high values of c becoming considerable: in order to determine which curve is applicable a larger range of experiment must be used.

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CXXXII. NORMAL VARIATIONS OF THE INORGANIC PHOSPHATE OF BLOOD.

By ROBERT EMLYN HAVARD and GEORGE ADAM REAY.

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(*Received September 23rd, 1925.*)

Consensus of experimental data, obtained from a large number of individual subjects, goes to show that the normal value for the inorganic phosphate of whole blood in man ranges from 1·9 mg. P to 3·8 mg. P per 100 cc. [de Wesselow, 1924], but singularly scant attention has been paid to the normal variations in the individual subject. One may not assume, as in the case of blood sugar, that in conditions generally considered to be normal, the inorganic phosphate remains absolutely steady, and that the taking of one sample will give a reliable figure.

In the course of experiments on the changes in blood phosphate under various physiological conditions, we investigated this question, and obtained results which it seems important to bear in mind in any similar work in the future, and also in the interpretation of some observations in the past, where the principle of ensuring a good normal does not seem to have been rigorously observed.

Method.

The method employed was a micro-adaptation of Briggs' modification of the Bell-Doisy technique [Briggs, 1922]. We consider that this adaptation, involving as it does the taking of only 0·5 cc. of blood, may have some clinical use. Moreover, in physiological experiments where a long series of samples is required, its convenience will be obvious.

Fifteen drops of blood are drawn from a pricked finger into a small crucible containing 0·5 mg. of sodium oxalate. 0·5 cc. of the blood is immediately delivered from an Ostwald pipette into a 6 cc. sampling tube containing 3·5 cc. of 0·5 % trichloroacetic acid (accurately measured). This concentration of acid has been shown to inactivate a phosphoric esterase in blood, which would otherwise give high figures for the inorganic phosphate [Martland, Hansman and Robison, 1924]. The blood is laked by inverting the tube once or twice, closed by the thumb, the proteins precipitated by the addition of 1 cc. of 22 % trichloroacetic acid, and the tube well shaken. Vigorous shaking is necessary to ensure a clear protein-free filtrate. The proteins are filtered off through a 6 cm. Whatman No. 50 filter paper, the filtrate, generally about 3 cc., being collected in a test tube. The inorganic phosphate in the filtrate is estimated on the same day, since acid hydrolysis gives appreciable errors if analysis be further delayed.

Small tubes (9 cm. long, and 0·7 cm. in diameter), graduated to hold 1·5 cc., are used as standard "flasks."

To 1 cc. of blood filtrate are added 0·1 cc. of Briggs' molybdate reagent; 0·05 cc. of Briggs' quinol reagent; 0·05 cc. of Briggs' sulphite reagent, and the whole made up to the 1·5 cc. mark. This is done in duplicate for each blood sample.

As standard 0·5 cc. of Briggs' dilute phosphate standard (1 cc. = 0·01 mg. P) is used, with the addition of 0·2 cc. of 22 % trichloroacetic acid to ensure the same degree of acidity as in the blood filtrate.

The solutions are allowed to stand for the usual half hour and read against the standard set at 20 mm. in a microcolorimeter. We have found the Bausch and Lomb instrument to give excellent results. The average discrepancy between duplicates was under 1 %.

Normal Variations.

A large number of experiments were done in which the subject walked to the laboratory in the morning and remained seated during the experiment. Blood was immediately taken on arriving, and thereafter as a general rule at intervals of 20 minutes. It was found that the inorganic phosphate was very unsteady, varying sometimes by as much as 14 % between the taking of the first and second samples, and usually dropped about 11 %. A steadier normal had therefore to be obtained. The difficulty was solved by allowing the subject to sit at rest for about 1½ hours before taking blood, and then almost invariably the variation in blood phosphate between successive samples was found to be below 2 %, and in many cases was 0 %. The probable reason for this drop becomes evident in the light of our results on the influence of exercise on the inorganic phosphate of the blood [Havard and Reay, 1925]. We have shown that on resting after exercise a drop in the inorganic phosphate occurs to an extent which is dependent on the vigour of the exercise and on the state of training of the subject. After the mild exercise of walking, the curve reaches in about 1½ hours a flat minimum, which provides a suitable base line from which to measure the effects of any superimposed experimental condition. This minimum contrasts well with the sharp minimum followed by a rapid rise obtained after vigorous exercise.

In Fig. 1 curves *C* and *D* show the steady normal which may be obtained by the subject resting. Curve *B* shows that even if the subject only moves about doing laboratory work, the phosphate may show greater variation.

We have observed on several occasions that a definite rise has taken place in blood phosphate about midday. This increase may amount to 16 % above the morning level (Fig. 1, Curve *A*, and Fig. 2, Curve *A*). This may possibly be correlated with the afternoon rise in phosphate excretion, investigated by Fiske [1921]. On other occasions, however, this rise in blood phosphate was absent, and in one experiment (Fig. 1, Curve *D*) a drop of 13 % was noted between 2 p.m. and 4 p.m.

Much further work is required to elucidate the cause of these variations, which probably reflect some alteration in general metabolism.

Since these irregular changes cannot be predicted, the morning seems to be the time most suitable for experiments, and results obtained in the afternoon must be interpreted with caution.

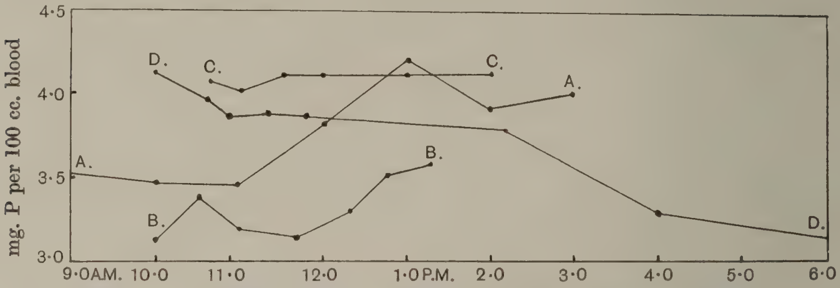

Fig. 1. A.—[R.E.H.] Rest. B.—[R.E.H.] Ordinary Laboratory work. C.—[E.W.] Rest. D.—[R.E.H.] Rest.

In order, therefore, to obtain a good normal blood phosphate for experimental work the subject should be seated in the laboratory as early in the day as possible to allow his blood phosphate to settle to a constant level and the experiment should be concluded as soon as possible in order to avoid complications due to the afternoon variations. We do not claim that a steady normal value will invariably be produced even by taking these precautions, but the value is sufficiently steady over two to three hours to make any experimental change obtained quite unmistakeable.

Influence of Sleep.

Although much work has been done on the influence of sleep on the constituents of blood and urine, it appears that only two previous observations on blood phosphate have been made, viz., by Gollwitzer-Meier and Kroetz [1924]; and by Haldane, Wigglesworth, and Woodrow [1924].

Rises in inorganic phosphate of 20 to 50 % due to sleep were noted by these workers. We have confirmed and extended these observations in the course of three experiments on one subject, R.E.H.

Exp. 1 lasted for a period of 30 hours (Fig. 2, Curve *A*), during which time the subject fasted, and was at rest either on a bed or in a camp chair, so that all exercise effects were cut out. The average day level (10 a.m.–10 p.m.) for inorganic phosphate was 3·56 mg. P per 100 cc., whilst the average sleep level was 5·06 mg. P per 100 cc., *i.e.* 42 % higher.

The sleep figure is the average of three analyses:

2.40 a.m.	... ...	5·19 mg. P %
6.0 ,,	... ...	5·01 ,,
8.0 ,,	... ...	4·97 ,,

The inorganic phosphate therefore remains steady at the same high level during sleep.

In Exp. 2 the usual diurnal cycle was reversed, and after a sleepless night, the subject slept from 10 a.m. until 3.25 p.m. A rise of 19 % in the blood phosphate was noted during the sleep period (Fig. 2, Curve *B*).

In both these experiments the inorganic phosphate fell rapidly to the lower level after the subject awoke.

In a third experiment half an hour's sleep produced no rise in inorganic phosphate, so that probably some considerable time elapses before the sleep level is reached.

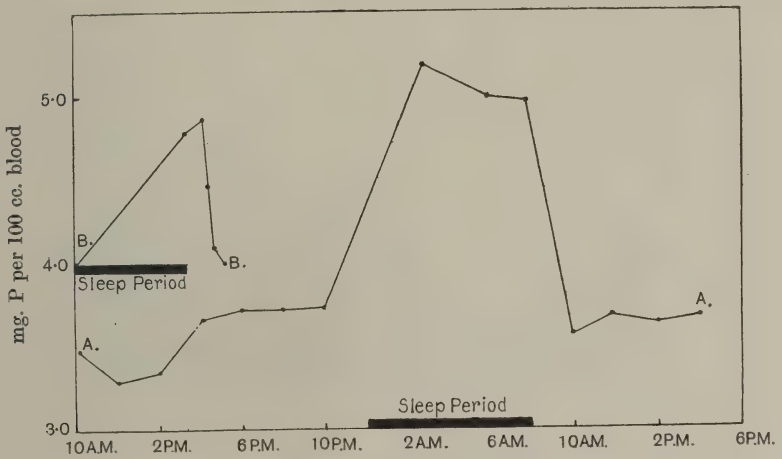

Fig. 2. Influence of sleep on inorganic P of blood.

Campbell and Webster [1922] and Kleitman [1923] have employed this reversal type of experiment in studying kidney excretion during sleep, but so far as we know it has not been carried out before for the inorganic phosphate of the blood. We have shown that the rise in inorganic phosphate of the blood cannot be separated from the phenomenon of sleep and that it is not an evidence of a diurnal physiological rhythm unconnected with sleep. This agrees with the observations by Campbell and Webster [1922] that there is an increased excretion of phosphate specifically associated with sleep. It has been shown that during sleep there is an increase in the alveolar CO_2 [Endres, 1923; Leathes, 1919] and a slightly decreased alkaline reserve and consequent increase in c_H [Collip, 1920]. These have been accounted for by assuming that the respiratory centre is less sensitive in sleep. A similar acidosis experimentally produced by Haldane, Wigglesworth and Woodrow [1924] by breathing CO_2 also caused a rise in the inorganic phosphate of the blood and an increased urinary excretion, and this has also been confirmed by us [Havard and Reay, 1925]. The evidence therefore points to CO_2 acidosis as the cause of the "sleep" rise in inorganic phosphate of the blood.

It is worthy of note that sleep is the only physiological condition associated with such a high blood phosphate in the adult. Moreover experimental CO_2 acidosis [Haldane, Wigglesworth and Woodrow, 1924] and phosphate ingestion [Wigglesworth and Woodrow, 1923] have not produced such high values as have been obtained in the case of sleep. It is true that Knipping [1922] has reported a value of 17·3 mg. P per 100 cc. of blood after "mental work." Since, however, he employed the uranium nitrate method, which gave him "normal" values as high as 8·9 mg. P per 100 cc., we do not consider these figures reliable. Moreover, in a single experiment of our own, on the influence of mental work, we could detect no change in the inorganic phosphate.

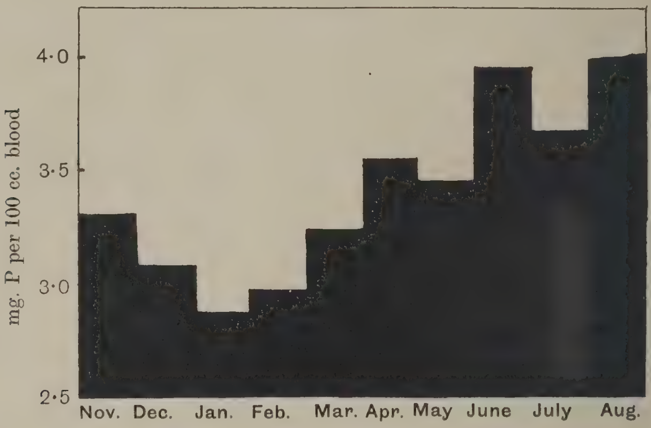

Fig. 3. Seasonal variation of inorganic P of blood.

SEASONAL VARIATIONS.

Between November 1924 and August 1925 we have made some 30 determinations of the normal morning blood phosphate at rest, on various subjects and on different days. The figures obtained show a definite seasonal variation of the type noted by Hess and Gutman [1922] in children, varying from an average of 2·9 mg. P per 100 cc. blood in January to an average of 4·0 mg. P per 100 cc. blood in August (Fig. 3). The irregularity noted during May, June and July, is due no doubt to the comparatively small number of samples. Three subjects, from whom blood has been obtained both in winter and summer, all show a marked increase in the normal for the summer months. Hess and Gutman consider the effect observed in children to be due to the increased intensity of the ultra-violet rays in summer, and it is probable that this also accounts for the changes occurring in the adult. The observation is of some interest in connection with the recent work on heliotherapy.

Summary.

1. A micro-adaptation of Briggs' method of blood inorganic phosphate determination is described which requires only 0·5 cc. of blood.

2. Precautions necessary in obtaining a steady "normal" blood phosphate figure are described and discussed.

3. The rise observed during sleep is confirmed and shown to occur even during sleep by day.

4. A seasonal variation is noted in the average normal blood phosphate of from 2·9 mg. P per 100 cc. in January to 4·0 mg. P per 100 cc. in August.

Our acknowledgements are due to Dr H. D. Kay for suggesting to us the micro-adaptation of Briggs' method and to Mr J. B. S. Haldane and Sir F. G. Hopkins for much helpful criticism and advice. One of us (R.E.H.) is indebted to the Department of Scientific and Industrial Research for a grant held during the course of the research.

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CXXXIII. A STUDY OF SOME BIOCHEMICAL COLOUR TESTS.

III. COLOUR REACTIONS ASSOCIATED WITH VITAMIN A.

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The familiar observation that liver oils give a transient purple colour on treatment with sulphuric acid acquired a new significance when Drummond and Watson [1922] demonstrated the close relationship between the presence of this reaction and the distribution of vitamin A. These observations have been confirmed by Poulsson and Weidemann [1923] and by Sjorslev [1924], although the specificity of the test has not yet been directly established.

The following work was undertaken to ascertain as far as possible the connection between the vitamin and the pigment; to see if the test would afford assistance in the isolation of the vitamin; and to obtain a colorimetric method for determining the quantitative distribution of the vitamin.

Since strong sulphuric acid under varying conditions produces a variety of colours with liver oils, and as only one of these, namely, the deep violet shade, appears to be associated with the vitamin, the test as performed and interpreted by the previous writers will be referred to in the present paper as the *Drummond* test.

THE NATURE OF THE PIGMENT PRODUCED IN THE DRUMMOND TEST.

The usual method of carrying out the test is to dissolve a drop or two of the liver oil in about 5 cc. of an anhydrous fat solvent. Addition of a drop of concentrated sulphuric acid followed by gentle agitation produces a deep violet colour if the test be positive. This colour soon fades, or is replaced by a red or brown shade. The violet colour is the essential part of the test.

That the actual pigment produced is independent of the nature of the solvent is shown by the similarity of the colour spectrum obtained in different solvents, or, in the absence of a solvent, by the direct addition of a dehydrating agent to the oil.

The intensity and rate of colour formation varies in different solvents approximately in the following order of decreasing response: xylene, toluene, benzene, light petroleum, "liquid paraffin," chloroform, carbon tetrachloride, carbon disulphide, ethyl ether. This was found to be due chiefly, but not entirely, to the greater water content of the less effective solvents. The colour test, like many sterol reactions, is extremely sensitive towards water.

In order to obtain standard conditions for carrying out the test the various factors concerned were investigated in turn.

Selection of a Solvent.

Light petroleum, B.P. 40–60°, was eventually selected as a solvent, owing to cheapness and other advantages which will appear later.

The light petroleum was dried by being shaken thoroughly with one-third of its volume of pure concentrated sulphuric acid, remaining in contact with the acid for some days. This process was repeated three times. It was then stored in a capped bottle, which had previously been dried in an air oven. It is essential for the success of the work that the solvent be free from water.

Selection of a Condensing Agent.

Strong sulphuric acid being obviously unsuitable, owing to the difficulty of controlling its action, other agents were tried including dry hydrochloric acid gas, trichloroacetic acid, zinc chloride and phosphorus pentoxide. With the last of these it was observed that the pigment formed did not go into solution in the body of the solvent but was adsorbed by the pentoxide and gradually subsided from the mixture as a dark purple precipitate; this suggested a method for the separation of the pigment.

The Phosphorus Pentoxide Test.

1. About a gram of phosphorus pentoxide is added to 5 cc. of the oil to be tested, either undiluted or dissolved in dry light petroleum, employing a well-dried tube. On gentle shaking, a deep violet colour develops if the oil be positive to the Drummond test. The colour slowly changes to a muddy brown depending on the amount of moisture originally present in the oil.
2. A drop of the oil to be examined is let fall on to the centre of a small heap of phosphorus pentoxide on a dry filter paper. If the test be positive a purple develops in a minute, and persists until the pentoxide has begun to deliquesce. Oils negative to the Drummond test give only yellowish colorations. The spectrum of the pigment suspension in dry benzene shows a well-marked band in the orange, 650–700$\mu\mu$.

Better results are obtained when the pentoxide is added directly to the oil and not to dilute solutions of the oil in an appropriate solvent. This appears to be due to the necessity of oxidation for the development of the pigment. Ordinary specimens of liver oil are able to effect this oxidation if present in sufficient amount. The peroxide value of liver oils is often high; some specimens can replace effectively the oxidised turpentine or hydrogen peroxide as a source of oxygen in the guaiacum test.

When the pentoxide test is applied to a weak solution of the oil, the development of the colour may be hastened by the addition of a suitable oxidising agent such as dry benzoyl peroxide. This difficulty does not arise in the original liver oil test as the sulphuric acid is able both to condense and to oxidise.

The Isolation of the Pigment in the Phosphorus Pentoxide Test.

It appears probable that the pigment obtained by the action of phosphorus pentoxide is the same as the transient violet compound formed in the Drummond test, and in the more recent tests introduced by Rosenheim and Drummond [1925]. The pigment formed in the pentoxide test can be easily separated from the oil by the following method: 50 g. of phosphorus pentoxide powder are rapidly ground up with 50 cc. of an active liver oil. The deep violet colour which develops reaches a maximum in about seven minutes. The mixture is then transferred to a set of *dry* centrifuge tubes and rapidly centrifuged. The pentoxide and adsorbed pigment separate, leaving a clear supernatant layer. This layer is poured off and the pigment precipitate washed while in the tube by means of dry light petroleum, and again centrifuged. This procedure is continued until the washings no longer give the Liebermann-Burchard test for sterols. The precipitates are then suspended in the light petroleum and poured into a litre flask three-quarters full of water, the tubes of the centrifuge being rinsed out with light petroleum.

The pentoxide with the adsorbed pigment is immediately hydrolysed, and the components of the pigment can be extracted from the solution by means of light petroleum by filling up the flask so that the solvent layer rises into the neck, where it separates on standing for a couple of days.

Notes on the Preparation. 1. Since water immediately decomposes the pigment great care must be taken throughout the preparation to exclude moisture. All the apparatus in contact with the pigment must be dried beforehand in an air-oven and stored in a desiccator. The presence of moisture is shown by the change in colour of the precipitate from purple to muddy brown.

2. Whitby's delicate test for sterols [1923] cannot be used in testing the washings, as the formaldehyde interacts with some unsaturated constituent of the light petroleum and obscures the test.

3. Unless the reagents are free from moisture it is not possible to wash the pigment precipitate free from sterol, since a moist solvent is able to decompose the pigment with the liberation of a substance capable of giving the general sterol reactions.

4. With a rapid centrifuge it does not appear necessary to seal the tubes, provided that the necks be kept quite free from any splashes of pentoxide.

5. Light petroleum is a particularly convenient solvent for washing the pigment precipitates since it can be separated very rapidly from the heavy

precipitate, and since it does not tend to form stable emulsions with the pigment components when added to water, as happens with the aromatic solvents, notably benzene.

The Character of the Pigment Constituents after Hydrolysis.

After evaporation of the light petroleum solvent the pigment constituents are left as a pale yellow oil. This oil gives the general sterol colour tests but does not give the Drummond test, nor the colour reaction with phosphorus pentoxide, nor the various polyphenol colour reactions described later in the paper.

The oil showed no signs of crystal formation on being kept for some weeks *in vacuo* at low temperature.

Before attempting any detailed chemical investigation of the residue, its growth-promoting effect was examined according to the method of Zilva and Miura [1921], the actual dietary having been previously tested in connection with other work in this laboratory [Pringle, 1925].

Six rats of comparable age and weight (25–55 g.) were employed (Fig. 1). The three controls were fed on the standard deficient dietary, with 20 mg. of cod-liver oil per rat per diem supplied by hand as the source of vitamin A. The second set of rats was fed on the same diet except that the pigment extract from an equivalent amount of oil was given instead of the oil. These rats had been on the deficient diet for three weeks previous to the experiment. Apart from an initial rise, the pigment extract showed no growth-promoting properties during a period of 20 days, after which the daily dose was increased to 0·1 g. with no appreciable result. This indicates either that the pigment is not intimately associated with the vitamin or that the process of condensation and subsequent hydrolysis changes the biochemical character of the material.

The latter alternative is supported by the fact that the final preparations no longer give the characteristic colour reactions.

THE EFFECT OF THE REMOVAL OF THE PIGMENT ON THE GROWTH-PROMOTING PROPERTIES OF COD-LIVER OIL.

Samples of cod-liver oil were treated with phosphorus pentoxide and centrifuged in the manner described until they no longer gave any colour with the reagent. The oils were then freed from excess of pentoxide by sedimentation during a period of one week.

These depigmented oils gave neither the Drummond test nor any of the colour reactions described in the present paper. The depigmented oil was administered in quantities of 20 mg. per rat per diem to each of the three rats which had been on the diet deficient in vitamin A supplemented by the pigment extract in the previous experiment, the pigment extract being discontinued during the administration of the depigmented oil.

No growth-promoting effect could be observed during the period when the depigmented oil was given, which supports the conclusion that the vitamin is a constituent of the pigment precursor. The potency of the oil used was shown by the control experiments.

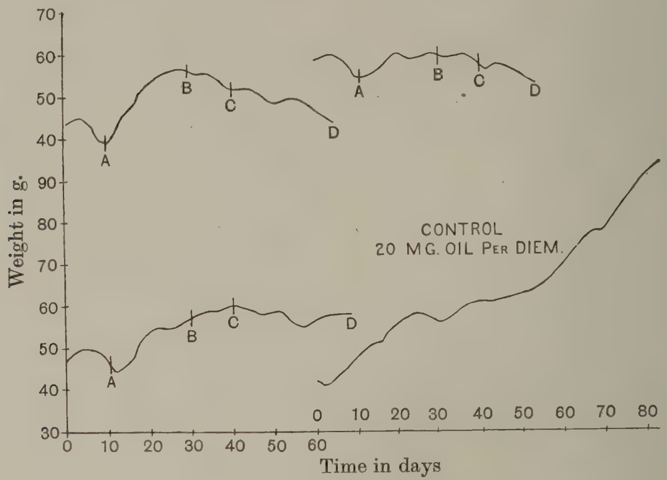

Fig 1.

A—B = Pigment equivalent of 20 mg. oil per rat p.d.
B—C = ,, ,, 100 ,,
C—D = De-pigmented oil, 20 mg. per rat p.d.

Colour Reactions suitable for the Colorimetric Estimation of Vitamin A.

If the pigment occurring in the Drummond and the phosphorus pentoxide tests be produced by a condensation between the vitamin and a sterol already present in the oil it should be possible to modify the test by replacing either component.

This has been done by Harden and Robison [1923], who have shown that small quantities of furfuraldehyde added to solutions of cholesterol, or to inactive oils containing cholesterol, react on treatment with strong sulphuric acid in a manner resembling that of liver oils.

The reaction appears to be similar in character to that devised by Whitby [1923] for the detection of sterols.

It has been suggested that the vitamin responsible for the colour test may itself be an aldehyde, but Drummond has pointed out that this is unlikely on account of the stability of the vitamin to the process of saponification, although it must be remembered that the vitamin may resemble the non-reducing disaccharides in being relatively stable in alkaline solution but readily undergoing decomposition in acid solution with the production of aldehydes.

The converse of the test does not yet appear to have been investigated; that is to say, the effect of the addition of sterols and polyphenols to solutions containing vitamin A before treatment with the condensing agent.

No difference was observed when excess of cholesterol was dissolved in a liver oil preparatory to carrying out the vitamin colour tests, presumably because the quantity of sterol naturally present was sufficient to give the maximum colour.

The effect of various polyphenols was then examined in the hope that they might interact with the vitamin to produce soluble pigments similar to those obtained by condensing pyrogallol with various aldehydes [Fearon, 1919].

The Condensing Agent.

The condensing agent must be of sufficient strength to bring about the reaction, without being in such concentration that it is able to produce a colour with the oil in the absence of the phenol. The condensing agent must also be able to hold the pigment in solution.

An entirely satisfactory result was obtained by employing a 12 % solution of dry trichloroacetic acid in dry light petroleum (B.P. 40–60°). This can conveniently be prepared by diluting a saturated solution of dry trichloroacetic acid in dry light petroleum (approximately 49 % at laboratory temperature) with three times its volume of light petroleum. Both the stock solution and the reagent should remain perfectly clear. They appear to keep indefinitely in the absence of moisture.

Pyrogallol, phloroglucinol, α-naphthol, and substituted phenols, such as vanillin and amidol, all give definite colours with liver oils in presence of the condensing agent.

Pyrogallol is probably the most suitable. It gives a bright rose colour with active oils. The colour is stable for a long period, and can be diluted with light petroleum for colorimetric comparison. It can be closely matched by a buffer solution containing phenol red.

The Pyrogallol Test for Vitamin A.

One or two drops of the oil, or dry ethereal solution of the substance to be examined, are placed in a dry 10 or 25 cc. flask. A few dry crystals of pyrogallol are added, and then 5 cc. of the condensing agent (12 % trichloroacetic acid in dry light petroleum). The contents are well shaken. A bluish pink colour slowly develops if the test be positive, changing to deep rose, and reaching its maximum intensity in 15 to 20 minutes.

Notes on the Test. 1. The rate of the development of the colour may be hastened greatly by warming the flask for a few seconds in a boiling water-bath. When the light petroleum begins to boil vigorously the flask should be removed, otherwise the liquid is apt to become cloudy.

2. Oxidation is necessary for the production of the colour. This is brought about partly by shaking the flask, and partly by peroxides already present in the oil. Some oils are very slow in producing a colour. This can be corrected by the addition of a very small quantity of dry benzoyl peroxide before

warming the solution. Excess of the peroxide must be avoided in colorimetric work, as it gives a yellow shade with the reagent on standing for some time.

3. As it is difficult to find a stable anhydrous solvent for pyrogallol which will not interact with the other reagents the pyrogallol is added in the solid form. Since it is only sparingly soluble in the acid light petroleum, the mixture must be well shaken at intervals.

4. Once formed, the colour is stable and will remain for days if the flask be properly closed. It can be diluted with dry light petroleum or with the condensing agent.

5. When the colour has developed, the contents of the flask should be perfectly clear, except for the undissolved crystals of pyrogallol at the bottom.

If the liquid is opalescent it indicates moisture. This sometimes may be removed by adding more of the condensing agent, shaking the flask and allowing it to stand for some time.

The Specificity of the Test.

In distribution and intensity, the pyrogallol test closely follows the Drummond and the phosphorus pentoxide tests. Oils inactivated by aeration, inactive vegetable oils, and hardened fats give no colour on standing in contact with the reagents for days, or, at most, a muddy yellow colour if an excess of the oil be taken.

It has not been found possible to reproduce the reaction by the addition of the commoner aldehydes and ketones, with and without cholesterol, to (1) medicinal petroleum, (2) aerated liver oil, (3) vegetable oil. The reaction with benzaldehyde most nearly resembles that of an oil containing vitamin A. The colour, however, is orange-red and soon fades.

Owing to the lack of success in the attempt to obtain a biologically active preparation from the pigment separated by the phosphorus pentoxide method no effort was made to isolate the pigment formed in the pyrogallol test.

It is of interest to note that the pyrogallol test is not given by "ostelin," "oxy-cholesterol," radiated cholesterol, or radiated hardened fat, which may be of significance in the differentiation between the fat-soluble vitamins.

CONCLUSIONS.

1. Phosphorus pentoxide forms a deep violet colour on addition to oils containing vitamin A.

2. The pigment may be completely separated from the oil leaving a residue deficient in growth-promoting properties.

3. The vitamin enters into the formation of the pigment, probably by condensation with a sterol.

4. It has not been found possible to recover the vitamin from the products of hydrolysis of the pigment.

5. Using a 12 % solution of trichloroacetic acid in dry light petroleum as a condensing agent, pyrogallol and other polyphenols interact with oils containing vitamin A to give stable pigments which are suitable for colorimetry.

I am indebted to Professor Sydney Young, F.R.S., for some chemically pure solvents, and to Mr H. Jephcott, and Messrs the Glaxo Company of London for their kindness in supplying me with information and with specimens of "ostelin." In the nutritional portion of the work I have been considerably assisted by W. Briggs of this department.

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CXXXIV. THE DISTRIBUTION OF PHOSPHORUS AND FAT IN THE RESTING AND FATIGUED MUSCLE OF THE CAT, WITH A NOTE ON THE PARTITION OF PHOSPHORUS IN THE BLOOD.

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Costantino [1912, 1914, 1917] was one of the first to attempt a more thorough study of the partition of the element phosphorus in muscle than had previously been undertaken. He recognised the presence of phosphatide, inorganic phosphate, protein phosphorus and an indefinite group of acid-soluble organic compounds. Further, he found that in the dog exercise produced an increase in the inorganic phosphate, but did not alter the total or extractive phosphorus, *i.e.* there was a change in the distribution but not in the total amount present. In the rabbit, however, no such alteration took place.

The work of Embden [1912–22] and his co-workers on the very labile chemistry of "lactacidogen" has rendered the previous determinations of the inorganic and total acid-soluble fractions of little or no value, owing to the rapidity with which this substance is split up or re-synthesised according to whether the muscle is contracting or returning to its resting phase.

In recent times the chief interest in the rôle of phosphorus in muscle metabolism has centred round the hexosediphosphoric ester found by Embden in muscle and now identified with the hexosediphosphoric ester previously demonstrated by Harden and Young [1911] in yeast juice. Of the significance of the other phosphorus compounds very little is known.

The occurrence in the animal body of organic compounds linked with phosphoric acid has given rise to much surmise. Indeed Meyerhof [1924] has put forward the hypothesis that this linkage renders the organic radicle the more accessible to oxidation. Respiratory quotient determinations during prolonged exercise led Benedict and Cathcart [1913], and Krogh and Linhard [1920], to the conclusion that although the energy for muscle work is derived mainly from carbohydrate, yet it cannot be the sole source. On a normal diet, Furusawa [1925] found that the oxidation of fat could only be detected after the oxidation of about 300 g. carbohydrate. On a fatty diet with a basal respiratory quotient of 0·71 the excess metabolism due to a short period of exercise was that of carbohydrate only, suggesting that fat was used only after conversion into carbohydrate. In long continued exercise on a fatty diet, fat was more quickly called upon than on a normal diet.

Palazzolo [1914] found that the muscle fat in frogs and hedgehogs was consumed even though the glycogen store was not exhausted. On the other hand, Leathes [1906] found no evidence of fat utilisation in muscles tetanised for several hours by stimulation of the sciatic nerve. Winfield [1915] using surviving muscles found that the fat content did not change after prolonged stimulation under anaerobic conditions nor after recovery in oxygen. Lafon [1913], working with the *levator labii superioris* of the horse and ass, found that more fat was removed from the blood when the muscle was working than when at rest. Bloor [1916, 1, 2; 1917; Bloor and MacPherson 1917], as a result of work on the distribution of fat and phosphatide in blood, came to the conclusion that, in general, wherever variations in the fat of the blood occur, there are found corresponding changes in the phosphatide, and at the same time he put forward the suggestion that phosphatide is an intermediate step in the utilisation of fat.

In view of the fact then that phosphorus may be linked with lipins and of the evidence that fat may be utilised as a source of energy, it was thought of interest to determine the fate of fat as well as of phosphatide in fatigued muscle.

General Plan of the Experiments.

The material used was the muscles of the domestic cat, an essentially carnivorous animal. In preference to prolonged stimulation under a general anaesthetic the animals were anaesthetised and decerebrated. After an interval blood was drawn from the left great saphenous vein, then the gastrocnemius, sometimes the triceps and biceps as well, were removed from the left side (first series). The sciatic nerve of the other limb was picked up in the gluteal region but was not cut. Faradic stimulation through the nerve was then carried out for varied periods using single induced shocks at first, then later tetanising the muscles by the application of more rapid stimuli. At the end of the stimulation blood was drawn from the intact right great saphenous vein. The gastrocnemius and the other muscles corresponding to the first series were then dissected out. By determining the distribution of phosphorus in the fractions to be described an attempt has been made to trace the gross changes of the phosphoric compounds during the different states of the musculature.

(*a*) The total P_2O_5 in the total acid-soluble extract provided an estimate of the changes of inorganic phosphate, lactacidogen and other unknown compounds.

(*b*) Determinations of the inorganic P_2O_5 were made on "*a*."

(*c*) Determinations of the inorganic P_2O_5 in muscle allowed to autolyse in an alkaline medium at 40° for $2\frac{1}{2}$ hours gave the lactacidogen plus inorganic P_2O_5 content. The former could then be calculated by the difference "*c*"–"*b*."

(*d*) The P_2O_5 of the unknown group was calculated from the difference "*a*"–"*b*."

(*e*) The P_2O_5 estimated in the fraction soluble in anhydrous ether and chloroform gave a rough method of calculating the changes in the phosphatide moiety.

(*f*) The nucleoprotein P_2O_5 was determined sometimes directly from the residue after the phosphatide and total acid-soluble extractions, sometimes indirectly by the difference of "*a*" plus "*e*" from the total P_2O_5.

The calculations throughout have been stated as percentages of the dried muscle. Such a precaution is necessary owing to the changes in the water relation which take place as the result of stimulation.

Experimental Procedure.

In order to reduce any effects due to recent ingestion of food, the animal received no food on the day previous to the experiment. When attempting the more prolonged operations of the later experiments, a complication, namely an excessive bronchial secretion, arose so frequently that it was found necessary to give an injection of 1/400 g. atropine sulphate hypodermically half an hour beforehand. Though an undesirable factor this allayed the excessive secretion without interfering with the muscular system [see Cushny, 1918]. The animal was anaesthetised with ether and decerebrated according to the method of Schmidt [1922].

After an interval of about 15 minutes, 5 cc. blood were drawn from the great saphenous vein of one limb, and added to 20 cc. of 5 % redistilled trichloroacetic acid[1] in a 25 cc. measuring flask. The flask was stoppered, shaken, and after 10 minutes the contents were filtered through a dry filter paper previously extracted with 1 % HCl.

The inorganic P_2O_5 was determined by the Briggs [1922] modification of the Bell-Doisy method [1920], 10 cc. of the filtrate being used. The total acid-soluble P_2O_5 was estimated colorimetrically on 5 cc. of the filtrate ashed with 0·3 cc. H_2SO_4 and 0·3 cc. HNO_3 in a micro-combustion tube. For the formation of the ammonium phosphomolybdate 2 cc. of a 5 % ammonium molybdate solution were used. The other reagents were as described in the original papers cited. The possible interference of the trichloroacetic acid and sulphuric acid used was tested. $N/2$ sulphuric acid and $N/10$ trichloroacetic acid using a 5 % ammonium molybdate solution to produce the phosphomolybdate had no effect on the colour production[2].

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[1] Martland and Robison [1924] found in some samples of trichloroacetic acid an impurity which produced a blue colour with the reagents used. Although this factor did not occur in the experiments described, the precaution of redistilling the reagent suggested by these authors was adopted.

[2] Stanford and Wheatley [1925] have recently found the same limiting acid concentrations. The present writer observed that allowing a period of 8–12 hours for colour production accurate readings were obtained with phosphate solutions even when the percentage difference was 50–100 %.

The residue from the above estimations together with the precipitated protein was oxidised with 7 cc. H_2SO_4 and 5–7 cc. HNO_3 and the total P_2O_5 calculated.

In these and subsequent estimations where it was desired to precipitate the phosphorus and weigh as phosphomolybdate, the precaution had to be taken, prior to precipitation, of adding an excess of HNO_3 (5 cc.) to the neutralised acid mixture. A granular ammonium phosphomolybdate which quickly and cleanly precipitated out was formed.

On the same limb from which the blood was drawn the gastrocnemius was dissected out, the vessels ligated and the muscle quickly cleaned of adherent fat and fascial sheath, then immersed in liquid air till frozen. The frozen muscle was then broken into small pieces in a cold stone mortar and divided into four lots—*A*, *B*, *C* and *D*—which were quickly weighed, while still frozen, in small stoppered bottles. This immersion in liquid air inhibits the changes in the isolated muscle, particularly the breakdown of "lactacidogen." Though the density of the muscle is decreased by this process, yet the condition is standard throughout the experiment, and is preferable to the rapid evaporation which results from the exposure of a warm isolated muscle even during rapid weighing.

Lot A of about 2 g. was weighed, teased out with needles, then dried in an electric oven at 98° until constant in weight. If drying is carried beyond this point other changes in the tissue take place. The percentage of water was calculated and a total P_2O_5 estimation was made on this dried extract using Neumann's wet ash method.

In this connection it was found that if the 7 cc. H_2SO_4 and 5 cc. HNO_3 used in these experiments were allowed to remain for several days at room temperature in contact with the dried substance to which has been added about 10 cc. water, oxidation proceeded to a considerable extent, and when heating was finally employed the oxidation was rapidly completed, thus obviating the chance of loss by volatilisation.

Lot B, about 2–3 g., was first weighed, then rapidly ground in a mortar containing 5 % redistilled trichloroacetic acid with broken glass. It is essential that the division of the tissue be fine as otherwise the thorough extraction of the acid-soluble material is inhibited by the precipitated film of protein. After about 2 hours the protein precipitate is filtered off, and washed thoroughly up to a known volume (100–200 cc.) with 0·5 % trichloroacetic acid. The inorganic phosphorus content was determined by the Briggs [1922] method. The total P_2O_5 was estimated gravimetrically.

Lot C, 2–3 g., was weighed, then minced finely with scissors with one-fifth of its weight as volume of saturated $NaHCO_3$.

The adherent fluid on the scissors was washed off with 15 to 20 cc. mammalian Locke solution containing neither glucose nor $NaHCO_3$. The vessel with its contents was immersed in a water bath at 50° for a few minutes and then placed in an incubator at 38° for 2½ hours. Embden and Laquer [1921] have demonstrated that this alkaline medium allows the breakdown of the lactacidogen by the specific muscle enzyme to go to completion.

An excess of 5 % trichloroacetic acid was added to precipitate the protein completely and extract the acid-soluble material. After standing for about 2 hours the mixture was filtered and washed to a known volume with 0·5 % trichloroacetic acid. Estimations of the inorganic phosphorus represented the P_2O_5 of the inorganic phosphate originally present, plus the additional phosphate liberated from the lactacidogen.

Lot D, approximately 10 g., after being weighed was finely ground in a mortar with broken glass and 95 % alcohol as fluid. This finely ground suspension, after standing for a few days, was filtered through a paper extraction thimble suspended in the interior of an Erlenmeyer flask. The filtrate was washed with alcohol into an evaporating basin and taken down almost to dryness on a water-bath, then desiccated over H_2SO_4 *in vacuo*. Over the suspended thimble a reflux condenser was now fitted and the tissue extracted with 95 % alcohol for 12 hours on an electric hot plate kept at the lowest possible temperature commensurate with steady extraction. After extraction the alcohol was washed with ether into a second evaporating basin and a further 12 hours extraction with ether carried out.

This extract was added to the alcohol in the second basin, and the products of subsequent 12 hours extractions with 95 % alcohol and ether were also added. The total fluid was then evaporated to a few cc. and the final desiccation completed *in vacuo* over H_2SO_4. The extracts were exposed to the air as little as possible. After as complete a desiccation as is compatible with the preservation of phosphatides unchanged, at least as regards their solubility, the residues of basins 1 and 2 were extracted several times in the cold with anhydrous ether and chloroform; anhydrous ether alone is probably insufficient as kephalin is insoluble in it. These precautions as regards using anhydrous reagents were necessary, for any water remaining in the solvents permits of a partial solution of other compounds, particularly inorganic salts. The combined ether and chloroform extracts were then evaporated almost to dryness on a water-bath and desiccated *in vacuo* over H_2SO_4. The weight of this dried material gave the total fat and "lipoid" substance, whilst a colorimetric estimation of the P_2O_5 after wet ashing gave the P_2O_5 as combined in phosphatide. An acid mixture of 0·75 cc. H_2SO_4 and 0·5 cc. HNO_3 is sufficient to oxidise the fat in the material obtained from 2 g. muscle.

It was thought that possibly the procedure adopted did not permit of the extraction of the masked fat of Dormeyer [1897] although MacLean [1918] has stated that the ether-insoluble combination of phosphatide and protein is acted on by alcohol and the phosphatide, which can subsequently be extracted by ether, liberated. In order to test if any fat remains unextracted, the residue in some of the experiments was digested with 1 % pepsin in 0·2 % HCl at 40° for 3–4 days. At the end of that period the fluid was neutralised with NaOH, then washed repeatedly with ether in a separating cylinder and the combined extracts treated as in the previously described fat estimation. A mere trace of fat was obtained which was found each time to be equivalent to the fat content of the enzyme preparation used. The ether and alcoholic extractions therefore remove all the fat.

Nucleoprotein P_2O_5.

Costantino [1915] demonstrated that the phosphorus-containing protein compounds of smooth muscle were nucleoprotein. To determine the nature of the protein phosphorus in striated muscle the residue from the fat extractions was first treated with 1 % HCl, then, after standing 2–4 hours, the product was filtered and the residue thoroughly washed with distilled water till free from all acid-soluble material. An extraction of this residue with 1 % NaOH for 48 hours at 37° was made according to the directions of Plimmer and Scott [1908]. A total P_2O_5 estimation was done at this stage. The remainder was then treated with 2 cc. glacial acetic acid for every 100 cc. solution and the precipitate filtered off. Since the phosphoric acid is split off the phosphoprotein molecule by this digestion, an estimation of the inorganic P_2O_5 reveals its presence or absence. No phosphoprotein was ever found so that the total P_2O_5 of the extract was due to nucleoprotein.

Description of the Experiments.

It may be stated that before these experiments were carried out, many estimations of the phosphorus content of the muscle of the cat were made in duplicate and triplicate as a test of the method and of the constancy of the distribution of that element.

Exp. 1. The two gastrocnemii were removed as soon as the depth of anaesthesia permitted.

Exps. 2, 3 *and* 4. In these experiments the resting gastrocnemii were removed 15 minutes after decerebration. The sciatic nerve in each case was picked up in the gluteal region of the other limb, and, for the first 10 minutes, stimulated with single induced shocks, then subsequently the muscles supplied by the nerve were tetanised by means of a Neef's hammer for periods of 15 to 50 minutes.

Exp. 5. After decerebration the resting gastrocnemius was allowed to remain *in situ* throughout the stimulation period of its fellow.

In Exps. 1 to 5, no atropine was injected. Nucleoprotein P_2O_5 was estimated directly. In all the experiments described, stimulation was carried on till the last moment prior to the actual dissection of the muscle.

Exp. 7. Blood was drawn from the great saphenous vein 15 minutes after decerebration. The lateral and medial cords of the brachial plexus on the right side were cut. Stimulation was started after the three muscles on the left side were removed and was continued for 45 minutes at which point the breathing stopped. A few minutes later the heart ceased beating. Stimulation was kept up till the moment of removal of the gastrocnemius. No atropine was used.

Table I. *Exps.* 1–5.

Number of experiment	1		2		3		4		5	
State of the muscle (gastrocnemius)	R.	R.	R.	F.	R.	F.	R.	F.	R.	F.
% dry matter	25·41	25·43	25·01	22·89	26·80	22·14	24·83	22·66	26·79	23·16
% water	74·59	74·57	74·99	77·11	73·20	77·86	75·17	77·34	77·86	76·84
Phosphatide P_2O_5 % of dry matter	0·25	0·24	0·19	0·17	0·29	0·28	0·27	0·28	0·17	0·17
Total acid-soluble P_2O_5 % of dry matter	1·84	1·82	1·92	1·95	1·88	1·91	2·02	2·00	1·83	1·82
Inorganic phosphate P_2O_5 % of dry matter	0·88	0·88	0·97	1·20	1·00	1·19	1·05	1·24	0·79	0·97
Nucleoprotein P_2O_5 % of dry matter	0·17	0·15	0·16	0·10	0·17	0·17	0·09	0·12	0·14	0·11
Total P_2O_5 % of dry matter	2·24	2·22	2·25	2·24	2·33	2·32	2·41	2·43	2·13	2·12
Period of stimulation	Resting			50 mins.		20 mins.		30 mins.		15 mins.

R. = resting. F. = fatigued.

Table II. *Exp.* 7. *Female Cat.*

	Left gastroc. removed after decerebration but prior to stimulation of right	Right gastroc. stimulated for ¾ hour after decerebration. Animal died	Left triceps removed after decerebration but before stimulation of gastroc.	Right triceps removed after stimulation of gastroc. for ¾ hour	Left cephalohumeral muscle removed after decerebration but before stimulation of gastroc.	Right cephalohumeral muscle removed after stimulation of gastroc. for ¾ hr.
% dry matter	24·31	20·74	24·16	25·29	24·76	25·74
% water	75·69	79·26	75·84	74·71	75·24	74·26
Phosphatide P_2O_5 % of dry matter	0·30	0·30	0·34	0·35	—	—
Total acid-soluble P_2O_5 % of dry matter	—	—	—	—	—	—
Inorganic phosphate P_2O_5 % of dry matter	1·03	1·37	1·00	0·97	1·05	0·94
Inorganic phosphate plus lactacidogen P_2O_5 % of dry matter	1·51	1·50	1·45	1·42	—	1·42
Lactacidogen P_2O_5 % of dry matter	0·48	0·13	0·45	0·45	—	0·48
Acid-soluble organic P_2O_5 other than lactacidogen % of dry matter	—	—	—	—	—	—
Nucleoprotein P_2O_5 % of dry matter	—	—	—	—	—	—
Total P_2O_5 % of dry matter	2·39	2·40	2·32	2·30	2·29	2·31

Exp. 8. This experiment was in nature a basal one. Half an hour prior to the operation 1/400 g. atropine sulphate was injected. The animal was anaesthetised with ether and decerebrated. Fifteen minutes later 5 cc. blood were drawn from the great saphenous vein on the left side, then the gastrocnemius, triceps and biceps on the same side were removed. The nerve supply to the biceps on the right side was severed. After a period of 1¾ hours rest in this condition, 5 cc. blood were drawn from the inferior vena cava and the three corresponding muscles removed from the living animal.

Table III. *Exp.* 8. *Female Cat.* 2·3 *kg.*

	Left gastroc. removed ¼ hr. after decerebration	Right gastroc. removed 1¾ hr. after decerebration	Left triceps removed ¼ hr. after decerebration	Right triceps removed 1¾ hr. after decerebration	Left biceps removed ¼ hr. after decerebration	Right biceps removed 1¾ hr. after decerebration during which period its nerve was cut
% dry matter	24·17	24·77	24·71	25·65	25·26	26·00
% water	75·83	75·23	75·29	74·35	74·74	74·00
Phosphatide P_2O_5 % of dry matter	—	—	—	—	—	—
Total acid-soluble P_2O_5 % of dry matter	1·94	1·92	1·92	1·87	1·92	1·95
Inorganic phosphate P_2O_5 % of dry matter	0·92	0·89	0·95	0·91	0·88	0·82
Inorganic phosphate plus lactacidogen P_2O_5 % of dry matter	1·31	1·28	1·37	1·32	1·26	1·25
Lactacidogen P_2O_5 % of dry matter	0·39	0·39	0·42	0·41	0·38	0·43
Acid-soluble organic P_2O_5 other than lactacidogen % of dry matter	0·63	0·64	0·55	0·55	0·66	0·70
Nucleoprotein P_2O_5 % of dry matter	—	—	—	—	—	—
Total P_2O_5 % of dry matter	2·36	2·35	2·43	2·39	2·32	2·35

Exp. 9. The animal was given a hypodermic injection of 1/400 g. atropine sulphate. Twenty minutes after decerebration the gastrocnemius, triceps and biceps of the left side were removed. On the right side, the nerve to the biceps was cut, then the sciatic nerve on the same side was picked up in the gluteal region and stimulated, first by single induced shocks but later fatigued by a Neef's hammer. The load of 150 g. was fixed to the paw so that the gastrocnemius contracted against it. This faradic stimulation was maintained for 2¼ hours when the other three muscles were removed for analysis.

Table IV. *Exp.* 9. *Male Cat.* 3·5 *kg.*

	Left gastroc. removed after decerebration but prior to stimulation of right	Right gastroc. removed after 2¼ hrs. stimulation with a load of 150 g.	Left triceps removed after decerebration but prior to stimulation of the gastroc.	Right triceps removed after fatigue of the gastroc.	Left biceps removed after decerebration but before stimulation of the gastroc.	Right biceps remained *in situ* with its nerve cut during stimulation period of the gastroc.
% dry matter	25·62	22·47	25·12	25·42	26·36	26·72
% water	74·38	77·53	74·88	74·58	73·64	73·28
Phosphatide P_2O_5 % of dry matter	0·29	0·27	0·30	0·30	—	—
Total acid-soluble P_2O_5 % of dry matter	1·84	1·89	1·94	1·96	1·98	1·97
Inorganic phosphate P_2O_5 % of dry matter	0·87	1·17	0·98	0·87	1·00	0·88
Inorganic phosphate plus lactacidogen P_2O_5 % of dry matter	1·40	1·42	1·45	1·46	1·54	1·51
Lactacidogen P_2O_5 % of dry matter	0·53	0·25	0·47	0·59	0·54	0·63
Acid-soluble organic P_2O_5 other than lactacidogen % of dry matter	0·44	0·47	0·49	0·50	0·44	0·46
Nucleoprotein P_2O_5 % of dry matter	0·11	0·06	0·14	0·15	—	—
Total P_2O_5 % of dry matter	2·24	2·22	2·38	2·41	2·31	2·29

Exp. 10. 1/200 g. atropine sulphate was administered. 5 cc. blood were drawn after decerebration from the great saphenous vein of the left limb, then the gastrocnemius, biceps and triceps were removed. The right gastrocnemius was fatigued by stimulation of the sciatic as in the previous experiments. The load was again 150 g. After stimulation for 50 minutes, 5 cc. blood were drawn from the inferior vena cava; the right gastrocnemius, triceps and biceps were then removed, the latter having had its nerve supply cut during the period of fatigue.

Table V. *Exp.* 10. *Male Cat.* 2·85 *kg.*

	Left gastroc. removed after decerebration but prior to stimulation of the right	Right gastroc. removed after 50 mins. stimulation with load of 150 g.	Left triceps removed after decerebration but prior to stimulation of the gastroc.	Right triceps which remained *in situ* during stimulation of gastroc.	Left biceps removed after decerebration but prior to stimulation of the gastroc.	Right biceps removed after the stimulation of the gastroc. during which its nerve was cut
% dry matter	24·16	20·58	23·29	23·75	24·94	24·91
% water	75·84	79·42	76·71	76·25	75·06	75·09
Phosphatide P_2O_5 % of dry matter	0·20	0·19	0·21	0·22	—	—
Total acid-soluble P_2O_5 % of dry matter	1·94	1·96	2·04	2·08	1·88	1·86
Inorganic phosphate P_2O_5 % of dry matter	0·89	1·17	0·89	0·84	0·80	0·87
Inorganic phosphate plus lactacidogen P_2O_5 % of dry matter	1·45	1·48	1·44	1·45	1·28	1·25
Lactacidogen P_2O_5 % of dry matter	0·56	0·31	0·55	0·61	0·48	0·38
Acid-soluble organic P_2O_5 other than lactacidogen % of dry matter	0·49	0·48	0·60	0·63	0·60	0·61
Nucleoprotein P_2O_5 % of dry matter	0·19	0·14	0·14	0·12	0·11	0·13
Total P_2O_5 % of dry matter	2·33	2·29	2·39	2·42	2·23	2·24

Table VI. *Exp.* 11. *Male Cat.* 3·9 *kg.*

	Left gastroc. removed after decerebration but prior to stimulation of right	Right gastroc. removed after 1¾ hr. stimulation with load of 150 g.	Left triceps removed after decerebration but prior to stimulation of the right gastroc.	Right triceps removed after 1¾ hr. stimulation of the right gastroc. during which time its nerve was cut	Left biceps removed after decerebration but prior to stimulation of the right gastroc.	Right biceps removed after the 1¾ hr. stimulation of the gastroc. but with its nerve intact
% dry matter	24·48	23·75	23·47	24·37	24·31	25·35
% water	75·52	76·25	76·53	75·63	75·69	74·65
Phosphatide P_2O_5 % of dry matter	—	—	—	—	—	—
Total acid-soluble P_2O_5 % of dry matter	1·78	1·81	1·94	1·92	1·84	1·83
Inorganic phosphate P_2O_5 % of dry matter	0·91	1·12	0·99	0·83	0·93	0·79
Inorganic phosphate plus lactacidogen P_2O_5 % of dry matter	1·25	1·25	1·24	1·25	1·25	1·25
Lactacidogen P_2O_5 % of dry matter	0·34	0·13	0·25	0·42	0·32	0·46
Acid-soluble organic P_2O_5 other than lactacidogen % of dry matter	0·53	0·56	0·70	0·67	0·59	0·58
Nucleoprotein P_2O_5 % of dry matter	—	—	—	—	—	—
Total P_2O_5 % of dry matter	2·25	2·23	2·32	2·30	2·29	2·26

Exp. 11. 1/400 g. atropine sulphate was injected in this experiment. The blood was drawn before and after stimulation from the great saphenous veins of the left and right sides respectively. Of the resting muscles the triceps had its nerve supply cut whilst the right sciatic nerve was stimulated for 1¾ hours. In other respects the procedure was the same as in the previous experiment.

Discussion of the Results.

Constancy of Composition. Exp. 1 demonstrates that corresponding muscles under like conditions have the same distribution of phosphorus and the same water content. Many others of this type were carried out with like result. It may be assumed that equality of distribution is the rule.

The Water Relation of Striated Muscle. From Exp. 8, it was observed that the water content of an unexercised muscle remaining *in situ* under the conditions stated, for a period of 1¾ hours, decreased by 1 %, and further, this decrease was independent of the integrity of the nerve supply (motor). Several interpretations were possible. The surgical shock may have caused a slight but immediate rise in the water content, due to a dilatation of the blood system in the muscle, and 1–2 hours later this may have returned to normal. Again the "plastic tonus" of decerebration, developing subsequent to the removal of the first series of muscles, may have caused a decrease in the volume of blood in the remaining muscles.

The possibility that these changes may have been due to the exhibition of atropine was, in part, negatived by the fact that they were found in Exp. 7, where the drug was not administered.

Whatever the cause of this effect may be, it occurs in the resting triceps and biceps independent of any stimulation of the leg muscles. In one experiment alone (10), where 1/200 g. atropine sulphate had been injected, did the integrity of the nerve supply affect the result.

The 4–5 % increase in the water content of the muscle after fatigue agreed with the figure obtained by Barcroft and Kato [1916] although these authors stimulated for a period of 7¾ hours. The factor responsible for this swelling of the fatigued muscle is probably, in large part, the filling out of the collapsed capillary system during exercise [Krogh, 1919].

The Total Phosphorus Content of Muscle. Fatigue of muscles to the extent described left the total phosphorus practically unaltered.

Phosphorus in the Total Acid-soluble Fraction. This moiety remained apparently constant even after fatigue. The small changes noted, though within the range of experimental error, yet pointed consistently in the direction of a slight increase on fatigue (1–2 %).

Inorganic and Lactacidogen Phosphorus. This group remained the most constant in amount in the varied muscle states.

Inorganic Phosphate. The outstanding feature was the marked increase in the fatigued muscle due to the breakdown of "lactacidogen." In some of the experiments a 34 % increase was noted. From the figures in Table III, it was found that under the conditions described there was a consistent but small decrease of 2–4 % in the inorganic phosphate of resting muscles 1¾ hours after decerebration. This seemed to take place even although the motor nerve was severed.

Table VII. *Blood Analyses.*

Experiment	8		10	
Time drawn	¼ hour after decerebration	1¾ hour after decerebration	¼ hour after decerebration but before stimulation	After 50 minutes stimulation
Vessel blood drawn from	Great saphenous vein	Inferior vena cava	Great saphenous vein	Inferior vena cav
Inorganic phosphate P_2O_5 as mg. % whole blood	16·8	15·9	12·7	27·6
Total acid-soluble P_2O_5 in mg. % whole blood	33·4	34·1	35·4	48·6
Total P_2O_5 in mg. % whole blood	52·2	—	—	—

Experiment	11		7	
Time drawn	¼ hour after decerebration but before stimulation	After 1¾ hours' stimulation	¼ hour after decerebration but before stimulation	After 45 minutes stimulation hear had just stopped
Vessel blood drawn from	Great saphenous vein	Great saphenous vein	Great saphenous vein	Heart
Inorganic phosphate P_2O_5 as mg. % whole blood	13·2	23·5	26·2	48·6
Total acid-soluble P_2O_5 in mg. % whole blood	31·2	34·9	—	—
Total P_2O_5 in mg. % whole blood	42·4	53·5	—	—

On superimposing stimulation on these conditions, the effect on the resting muscles was still further to decrease the inorganic phosphate in some cases by as much as 11 %. With one exception (Exp. 10), similar results were found where the resting muscle had had its nerve severed. In Exp. 10 a 2 % increase was found in the biceps with its nerve cut. This effect may have been due to the relatively large dose of atropine administered.

The question of assessing to what extent changes in the blood phosphorus might affect the estimations of that element in muscle presented itself. On calculation it was found that only in the case of the oedematous fatigued muscle can the changes in the blood appreciably affect that of muscle, and even in this state of the muscle, the extent of the possible change comes within the range of error. Again the changes in the blood to some extent reflect the changes in the muscles.

"*Lactacidogen*" P_2O_5. Exp. 8 demonstrated that the content of lactacidogen remains practically constant. In the non-stimulated muscle, an increase of 0·38–0·43 % of the dried tissue was found in the resting biceps with the nerve supply cut. On superimposing stimulation on these experimental conditions, it was found that there was a very marked decrease of lactacidogen amounting to 60 % in some experiments. On the other hand, the effect of exercise was to cause an increase of 20–50 % in the other resting muscles. With the one exceptional case in Exp. 10, this increase was found to be independent of the integrity of the nerve supply.

This increase of lactacidogen in the resting musculature was interesting as suggesting a possible synthesis of that compound at the expense of the inorganic phosphate already present. Another explanation is possible, namely, that the effect of the surgical shock was immediate, wearing off 1–2 hours later, and that while it lasted, it caused a breakdown of lactacidogen with the liberation of inorganic phosphate.

Acid-soluble P_2O_5 *in Organic Form other than "Lactacidogen."* It was found that this moiety remained very constant in the resting phase and exercise left it practically unchanged. This fraction representing 25 % of the total P_2O_5 is unexplored.

Table VIII. *Fat Analyses.*

	Fat expressed as % of dried substance			
Exp.	Left gastrocnemius R.	Right gastrocnemius F.	Left triceps R.	Right triceps non-stimulated *in situ*
10	4·58	4·60	4·43	4·34
9	8·97	8·91	7·78	7·89
7	8·87	8·75	9·00	9·05
2	7·75	7·35	—	—

R. = resting. F. = fatigued.

Fat and Phosphatide Content of Muscle. From the analyses found in Table VIII it will be seen that in three out of the four experiments described, there has been a slight decrease on fatigue. In Exp. 10 the content remained practically constant. These differences however, though tending in the same direction, come so nearly within the range of experimental error, that a definite conclusion cannot be reached.

With regard to the phosphatide phosphorus, exercise for the periods described has not caused any change in its amount.

The data then show practically no evidence of the utilisation of fat or phosphatide in muscle work under the experimental conditions observed, but this does not negative the possibility that these substances may be utilised after the exhaustion of the available carbohydrate. In none of these experiments was the lactacidogen content of the fatigued muscle completely used up.

The Changes in the Distribution of the Phosphorus in the Blood. Kay [1925] has classified the phosphorus compounds of the blood under the following headings: inorganic phosphate, probably two hexosephosphoric esters, an adenine nucleotide [Jackson, 1924] and lecithin. Practically no change takes place in the blood unless muscular exercise is superimposed on the conditions described (Exp. 8, Table VII).

In assessing the changes that take place on fatigue, a number of factors tend to obscure the issue. Schneider and Havens [1915] found that the immediate effect of exercise was a concentration of the blood in the peripheral capillaries, but, in a few minutes the blood was diluted. The increased content of phosphorus in the blood found in Exp. 11 may be due to this peripheral concentration alone, or to this factor accompanied by an actual increase in the circulating phosphoric compounds. There is a very definite increase in the inorganic phosphate of the blood on exercise (see Exps. 7, 10 and 11). This is probably due to the washing out of that metabolite from the active muscles into the circulation. The total acid-soluble phosphorus increases to a small extent. This may be due either to the concentration of the blood or to an increase in the corpuscles, or to both factors. The authors mentioned above found an increased erythrocyte count, whilst Isaacs and Gorden [1924] found the red corpuscles unchanged after exercise. The increase in Exp. 10 is probably due to the fact that the blood was drawn after exercise from the abdominal veins where there may be some accumulation of cells.

The fact that the total acid-soluble P_2O_5 does not increase proportionally with the rise in inorganic phosphate, suggests either that the organic acid-soluble compounds have been removed in part, or have been split up in the circulation with the liberation of inorganic phosphate. The active muscle, having exhausted its reserve of lactacidogen, may call on the hexosephosphates or other phosphorus compounds of the blood, when no further synthesis from its own substance is taking place commensurate with the demands made.

SUMMARY.

1. An experimental procedure has been developed for the examination of the effect of fatigue on the phosphorus content of muscle.

2. A few suggestions relating to the estimation of phosphorus in different tissues and extracts have been put forward.

3. Muscle fatigue to the extent described appeared to cause:

(*a*) Practically no change in the total P_2O_5.

(*b*) An increase in the inorganic P_2O_5 of the stimulated muscle, the maximum increase found being 34 %. A decrease in the inorganic P_2O_5 of resting muscles remaining *in situ* during the stimulation period was found; the maximum decrease noted was 11 %.

(*c*) A decrease in the lactacidogen P_2O_5 in the fatigued muscle amounting in some experiments to 60 %. An increase in the lactacidogen P_2O_5 of 20–50 % was found in the muscles resting *in situ*.

(*d*) No alteration in the total amount of inorganic plus lactacidogen P_2O_5.

(*e*) Little or no effect on the organic acid-soluble P_2O_5 other than lactacidogen.

(*f*) A consistent but small increase (1–2 %) in the total acid-soluble fraction.

(*g*) No alteration in the phosphatide P_2O_5.

4. The effect of fatigue on the phosphorus compounds of the blood was found to cause:

(*a*) An increase of the inorganic P_2O_5. A 100 % increase was recorded after 50 minutes' stimulation.

(*b*) An increase in the total acid-soluble P_2O_5 (12–37 %).

(*c*) A decrease in the organic acid-soluble P_2O_5 (7–36 %).

5. An increase of 4–5 % in the water content was noted after stimulation of a muscle for 45 minutes.

6. The phosphorus-containing proteins of skeletal muscle belong to the nucleoprotein group.

7. An attempt to settle the question of the utilisation of fat and phosphatide in muscle work was undertaken with essentially a negative result. No "masked fat" was found to remain after a thorough alcoholic and ethereal extraction of the muscle tissue.

In conclusion I wish to express my thanks to Professor E. P. Cathcart not only for suggesting this study, but for his advice, criticism and inspiring enthusiasm throughout. To Professor D. Noël Paton for his kindly interest and advice in the operative technique, to Mr Watson and the others who so willingly gave of their time, and under whose licences the experiments were conducted, I desire to express my thanks.

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CXXXV. THE FATE IN THE ANIMAL BODY OF PHENYLSUCCINIC ACID AND β-PHENYLHEXOIC ACID.

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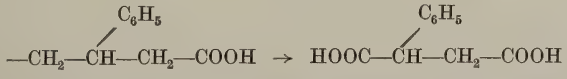
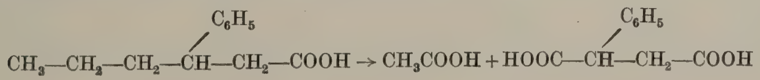
In a previous paper, Clutterbuck and Raper [1925] have shown that when the ammonium salts of fatty acids are oxidised with hydrogen peroxide, oxidation, besides taking place at the β-carbon atom, as described by Dakin [1908, 1, 2, 3], also takes place at the γ- and δ-carbon atoms. The γ- and δ-keto-acids formed in this way, on further oxidation, yield succinic acid. Since succinic acid occurs in the body in various tissues, and an enzyme, succinoxydon, capable of oxidising it, is widely distributed in animal tissues, it seemed possible that the process described above, which takes place *in vitro*, might be of biological importance. Little is known about the mode of production of succinic acid in the body and the experiments mentioned above suggested that oxidation of fatty acids at the γ- or δ-carbon atom might be one means by which succinic acid arises in metabolism. The experiments described in this paper are among several which have suggested themselves as likely to throw light on this hypothesis.

It was thought that if a fatty acid with a normal chain of four or more carbon atoms were substituted in the β-position by a phenyl group, so as to render the β-carbon atom less accessible to oxidation, then phenylsuccinic acid should be produced if γ oxidation, with removal of four carbon atoms, took place.



$$—CH_2—\overset{C_6H_5}{CH}—CH_2—COOH \rightarrow HOOC—\overset{C_6H_5}{CH}—CH_2—COOH$$

If phenylsuccinic acid is not easily oxidised in the body then it should be excreted in the urine if it were produced as pictured above. Administration of phenylsuccinic acid to a dog (dose 0·22 g. per kg.) showed in fact that it was not easily oxidised, since 61 % of the amount given was recovered from the urine. The main experiment was therefore undertaken. The acid chosen was β-phenylhexoic acid, which, if it were oxidised at the γ-carbon atom, would produce acetic acid and phenylsuccinic acid.



$$CH_3—CH_2—CH_2—\overset{C_6H_5}{CH}—CH_2—COOH \rightarrow CH_3COOH + HOOC—\overset{C_6H_5}{CH}—CH_2—COOH$$

On administering this acid subcutaneously in the form of its sodium salt, 45 % of it was recovered from the urine chiefly as a compound with glycuronic acid, but not a trace of phenylsuccinic acid could be detected. The amount of unoxidised acid was probably greater than this since the method of isolation of the acid from the urine is not strictly quantitative. It appears therefore that β-phenylhexoic acid is oxidised with difficulty in the body and that phenylsuccinic acid is not one of its oxidation products. It seems probable therefore that in this instance γ-oxidation does not take place. Other experiments which have been designed to answer the question are being taken in hand.

EXPERIMENTAL.

Phenylsuccinc Acid.

The specimen used[1] was prepared by the method of Lapworth and McRae [1922]. It was converted into the sodium salt and an amount of this containing 1·4 g. of the acid was injected subcutaneously, on each of two successive days, in 10 % solution into a dog weighing 6·5 kg. The urine was collected from the time of the first dose to 48 hours after the last dose had been given. The urine, concentrated on the water-bath, was acidified with phosphoric acid and shaken out with ether and then extracted with the same solvent for 18 hours in a continuous extractor. On removal of the ether a crystalline mass was obtained. This was extracted several times with warm light petroleum, to remove traces of higher fatty acids, dissolved in hot water, clarified with charcoal, evaporated to small bulk and left to crystallise. In this way 1·71 g. of practically pure phenylsuccinic acid was recovered, M.P. 167°, equivalent 96·9 (calc. 97). The ether extract of the urine obtained by continuous extraction contained a small amount of an acid much less soluble in cold ether than phenylsuccinic acid. It was separated from the latter acid by means of a little warm ether and melted at 140–2°. It was not phenaceturic acid since a mixed M.P. determination caused the M.P. to drop to 110–17°. The amount obtained was too small for further investigation. In this experiment, therefore, 61 % of the phenylsuccinic acid given was recovered from the urine. The recovered acid, examined in 4·3 % solution in alcohol, was optically inactive.

β-Phenylhexoic Acid.

This acid was prepared by condensing butyrophenone with bromoacetic ester in dry benzene solution using a modification of the Reformatsky [1887] and Lindenbaum [1917] methods.

The crude β-phenyl-β-n-propylhydracrylic ester thus obtained was gradually dissolved in sulphuric acid and poured on to ice [Schroeter, 1907, 1908]; β-n-propylcinnamic acid separated and was recrystallised from light petroleum. The acid was neutralised with sodium hydroxide and reduced in the usual way with sodium amalgam. The crude β-phenylhexoic acid was recovered by acidification with hydrochloric acid and extraction with ether. It was dried and fractionated *in vacuo*. The acid was collected at 187–9° (20 mm.). Equivalent 188·5 (calc. 192). The glycine compound was prepared in case it might be required for purposes of identification. The acid chloride was prepared from a small portion of the acid by the action of phosphorus trichloride and converted into the glycine compound by the Schotten-Baumann method. A little unchanged acid was removed from the product of this reaction by extraction with light petroleum and the β-phenylhexoylglycine crystallised from aqueous alcohol. M.P. 131°. (N = 5·7 %, $C_{14}H_{19}O_3N$ requires N = 5·6 %.)

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[1] This was kindly presented to us by Professor Lapworth.

The same dog which had received the phenylsuccinic acid was given, subcutaneously, 1·5 g. of the β-phenylhexoic acid as the sodium salt in 10 % solution on each of two successive days. It produced no ill effects. The urine, collected for the succeeding 72 hours after the first dose, was worked up as in the experiment with phenylsuccinic acid. The ether extract of the acidified urine on evaporation left a straw-coloured syrup. It was extracted several times with warm light petroleum and this removed some unchanged acid together with traces of higher fatty acids. The latter were removed as insoluble barium salts and the unchanged acid recovered by acidification and extraction with light petroleum. That portion of the original ether extract which was insoluble in light petroleum failed to crystallise and inoculation with a trace of phenylsuccinic acid failed to bring about crystallisation. Since it reduced Fehling's solution slightly on boiling, the presence of a glycuronic acid compound was suspected. The syrup was therefore decomposed by boiling for 6 hours with $N/50$ sulphuric acid and again extracted with ether. The greater part of the ether extract was soluble in light petroleum and consisted of unchanged β-phenylhexoic acid. That part which was insoluble in light petroleum was boiled with 20 % hydrochloric acid and yielded a little more acid soluble in light petroleum. In all 1·36 g. of unchanged acid was recovered. Equivalent 196 (calc. 192). The aqueous solution obtained by hydrolysis with $N/50$ sulphuric acid after ether extraction was neutralised with sodium hydroxide and concentrated to about a tenth its volume on the water-bath. The solution thus obtained reduced Fehling's solution strongly on heating, gave the orcinol reaction and a crystalline osazone which could not be recrystallised owing to decomposition. All these properties being characteristic of glycuronic acid there is no doubt that the unchanged acid was present in the urine largely as an ether-soluble glycuronic acid compound. The unchanged acid recovered, as described above, was optically inactive. It amounted to 45 % of that administered.

SUMMARY.

1. Phenylsuccinic acid administered subcutaneously to a dog was excreted unchanged in the urine to the extent of 61 %.

2. β-Phenylhexoic acid administered similarly was excreted partly unchanged and partly as a glycuronate; 45 % of the acid was recovered in this way. Phenylsuccinic acid was not detected as one of its oxidation products.

3. These observations indicate that β-phenylhexoic acid does not undergo γ-oxidation in the body since this should lead to the formation and excretion of phenylsuccinic acid.

We are indebted to the Government Grant Committee of the Royal Society for a grant in aid of the expenses of this investigation.

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CXXXVI. THE INFLUENCE OF THE ADMINISTRATION OF CERTAIN SALTS ON THE YIELD AND COMPOSITION OF MILK.

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(*Received October 8th, 1925.*)

INTRODUCTION.

It is a well-established fact that the calcium concentration of blood remains at a very constant level, and that no measurable effects are produced by feeding rations with abnormally high contents of calcium. Recently Stewart and Haldane [1924] have claimed that the calcium content of human blood serum can be altered, within limits, by the oral ingestion of large quantities of certain salts. The objects of the experiments recorded below were to ascertain whether similar alterations could be effected in the calcium content of the blood plasma of lactating cows, and to discover what alterations, if any, were induced in the composition of the milk by this means.

The animal selected for the experiments was a white Shorthorn heifer; she weighed approximately 500 kilograms, and at the commencement of the experiments was in her fifteenth week of lactation, yielding 13 lbs. of milk per day.

Each experimental period was of about one week's duration, the animal being given sufficient time between the experiments to allow of recovery to normal.

The salts used were: (1) for raising the plasma calcium content, calcium chloride and ammonium chloride; (2) for lowering the plasma calcium content, sodium bicarbonate and disodium hydrogen phosphate (Na_2HPO_4). The salts were given in solution as "drenches," the doses being spread over a period of six hours (10 a.m. to 4 p.m.).

During the experimental periods, the cow remained on a normal daily ration, which consisted of 3½ lbs. seeds hay, 30 lbs. silage, 20 lbs. marrow stem kale, 50 lbs. mangels, and 2½ lbs. concentrates. This ration contains approximately 4 ozs. of CaO and 3 ozs. of P_2O_5.

For convenience we have divided the results into two sections, the first showing the effect of the various salts on the calcium content of the blood plasma, and the second, their influence on the composition of the milk.

CALCIUM CONTENT OF BLOOD PLASMA.

The method of analysis employed has been published elsewhere [Little and Wright, 1925].

A summary of the results obtained is shown in Table I, and in Table II these results are compared with those obtained by Stewart and Haldane on a human subject.

Table I. *Alteration in the Calcium Content of Cow's Blood Plasma.*

Weight of cow, 500 kilograms.
Normal calcium content of plasma, 9·8 mg. per 100 g.

Experiment*			Blood sample taken	mg. Ca per 100 g. plasma
Day 1.	$CaCl_2$	6 oz.	Next morning	9·6
Day 2.	,,	12 oz.	,,	10·1
Day 1.	NH_4Cl	8 oz.	—	—
Day 2.	,,	16 oz.	6.0 p.m. same day	7·35
			Next morning	8·4
Day 1.	$NaHCO_3$	6 oz.	Next morning	9·8
Day 2.	,,	12 oz.	—	—
Day 3.	,,	24 oz.	5.0 p.m. same day	9·8
Day 1.	Na_2HPO_4	32 oz.	8.0 p.m. same day	9·0

* All doses given between 10.0 a.m. and 4.0 p.m.

Table II. *Comparison of Results.*

	Dose (g. per kg. body-weight)		Percentage rise or fall	
Salt	Human	Cow	Human	Cow
$CaCl_2$	0·30	0·36	+25	−2
	—	0·72	—	+3
NH_4Cl	0·25	0·48	+10	—
	—	0·96	—	−24
$NaHCO_3$	0·60	0·36	−20	±0
	—	0·72	—	—
	—	1·44	—	±0
Na_2HPO_4*	0·60	1·90	−20	−8

* Private communication from Dr J. B. S. Haldane.

It will be seen that none of the results is in agreement with those recorded by these two workers. The results are the more striking since in our experiments doses were given which were proportionately and actually far in excess of those quoted by them as adequate to produce marked alterations in the serum calcium content.

At present it is impossible to explain this discrepancy. The results obtained by us can hardly be attributed to analytical errors, for very great care was taken to guard against these: the quantities of plasma at our disposal (varying from 15 to 25 g.), and the good agreement of duplicate determinations were alone sufficient to preclude the possibility of errors of such magnitude. From this point of view the use of a cow as an experimental animal was of considerable advantage.

Further, the more complete results shown in Fig. 1 demonstrate the very consistent results obtained throughout each experimental period.

It appears possible that the different morphology of the alimentary tract of the cow and the different nature of the diet may be responsible for these contradictory results, though such an explanation cannot account for the action of the ammonium chloride. It is also possible that the cow has developed a more efficient mechanism for controlling the level of calcium in the blood, since she is liable to continual loss of large quantities of calcium in the milk. In any event, our work demonstrates that the calcium content of the blood plasma of a lactating cow cannot very easily be altered. This result is contrary to those obtained by Stewart and Haldane for the human subject.

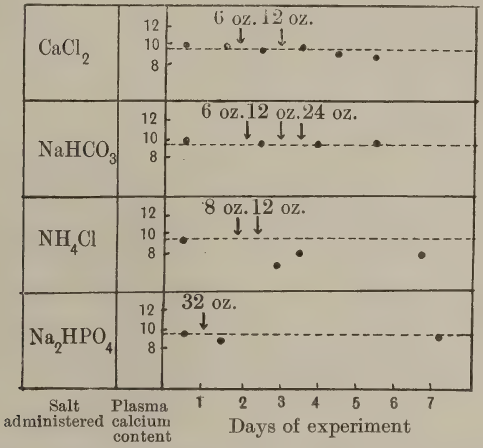

Fig. 1. Effect of administration of salts on the calcium content of the blood plasma.
(Dotted line represents normal value.)

YIELD AND COMPOSITION OF MILK.

In three of the four experimental periods (dosage with calcium chloride, sodium bicarbonate, and disodium hydrogen phosphate) there were no significant alterations either in the yield of milk or in those constituents of the milk which were estimated, *i.e.* fat, total protein, caseinogen, lactose, ash and calcium (Figs. 2 and 3). With regard to other inorganic constituents we have no direct evidence. Measurements have been made of the time of coagulation of milk with rennet, a reaction which we should expect to be a delicate measure of alterations in the "salt balance" in milk. In no case were there any significant alterations in renneting time. There were also no alterations in reaction, as judged by colorimetric tests with bromocresol purple indicator.

In the third period (dosage with ammonium chloride) the changes were profound. The following are the notes recorded during this experimental period.

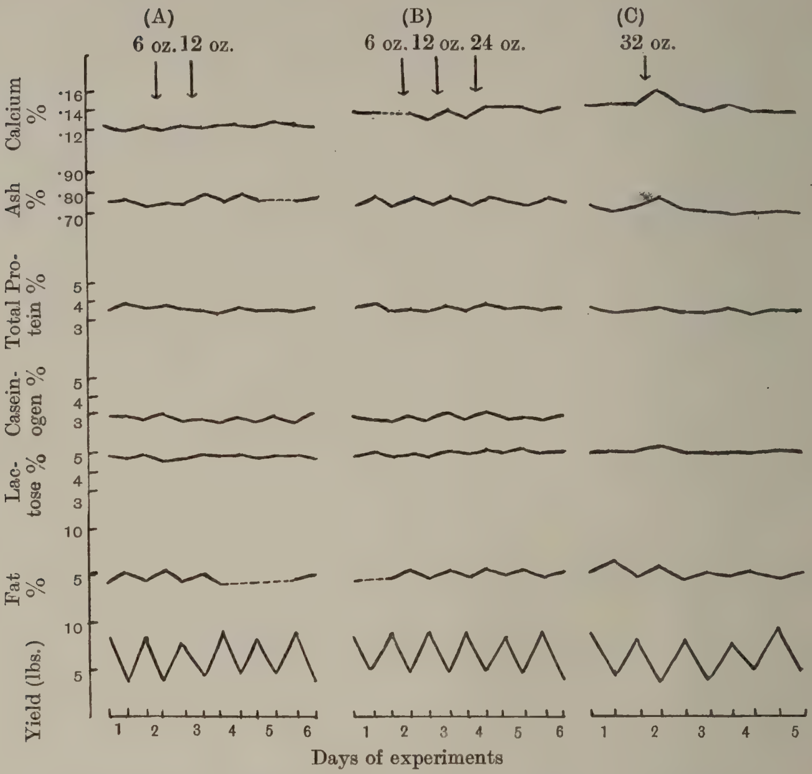

Fig. 2. Effect of administration of (A) $CaCl_2$, (B) $NaHCO_3$, (C) Na_2HPO_4, on the yield and composition of milk.

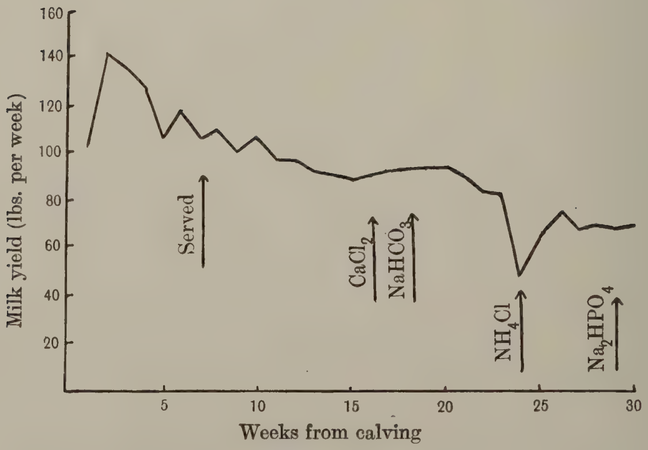

Fig. 3. Lactation curve, showing effect of administration of salts on milk yield.

"The first day was kept as a control, and no dose given.

"No adverse effects were observed after ingestion of 8 ozs. on the second day (March 17th, 1925).

"On the third day (March 18th) doses were given as follows: at 10 a.m. 6 ozs., at 12 noon 6 ozs., at 2 p.m. 4 ozs. At 3 p.m. the cow showed signs of discomfort. She refused food, was breathing rapidly, and would not rise. At 5.30 p.m. she was still unable to rise and was obviously distressed. At 7.30 she had risen but was not comfortable: there was profuse urination. The milk yield was reduced from 4 lbs. to less than ½ lb. The following morning (March 19th) she was up, but off her feed; her eyes were glazed, but sight appeared normal: the milk yield was almost entirely suppressed. The following day (March 20th) she appeared easier; breathing was normal: the eyes appeared weak, and the left eyelid was swollen. The milk yield was increased. From this time there was a gradual recovery both in condition and in milk yield."

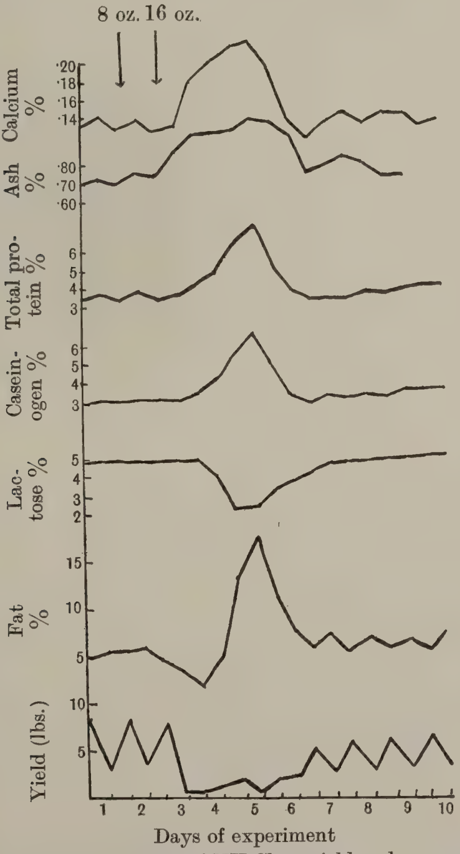

Fig. 4. Effect of administration of NH_4Cl on yield and composition of milk.

It will be noted that on the third day, after ingestion of the large dose (16 ozs.) of ammonium chloride, the milk yield fell to a fraction of the normal figure (Fig. 4). The composition of the milk, however, remained normal, except for the fat which was slightly low. During the next five milkings, the yield remained low (varying from $\frac{1}{2}$ to $1\frac{3}{4}$ lbs.), but the composition was entirely altered. The fat, protein and ash rose to a maximum at the time of the fourth milking (in which the fat reached 17 % and the protein 7·5 %), but subsequently returned to normal as the milk yield increased. The lactose on the other hand fell to a minimum at the third milking, and then slowly returned to a normal value.

The explanation of these facts is not clear, but the following suggestions are put forward to account for the observed results. The immediate consequence of the heavy dosage was a complete disorganisation of normal metabolism, which resulted in a partial suppression of milk yield, but which caused no alteration in the composition of the small quantity which was secreted. In the two following days, although the quantity of milk secreted had increased, the ammonium chloride exerted its influence as a dehydrating agent [Haldane, Hill and Luck, 1923], and caused by the removal of water a concentration of the constituents of the milk to the extent shown in Fig. 4. The lactose, which is easily diffusible, was re-absorbed into the blood stream, and actually appeared in the milk in lower concentration than normal.

The whole experiment indicates, however, that the methods employed are useless for the purpose for which this part of the work was undertaken, *i.e.* the correlation of the calcium content of blood and milk.

In the first three periods of the experiment (dosage with $CaCl_2$, $NaHCO_3$ and Na_2HPO_4), although very considerable quantities were given, the results proved negative: in the fourth period (dosage with NH_4Cl) the interference with normal metabolism was so great as to cause suppression of milk yield and gross alterations in composition, with consequent vitiation of results.

SUMMARY.

Attempts have been made to alter the calcium content of the blood plasma of a cow by means of large oral doses of certain salts, and the effect of these salts on the composition of the milk has been studied.

The results indicate (*a*) that it is extremely difficult to alter the plasma calcium content by these means, and (*b*) that it is impossible by such means to effect any alteration in the composition of the milk.

With ammonium chloride (16 ozs.) the severe disturbance of normal metabolism, and the dehydrating action of the salt, caused an almost complete suppression of milk yield, accompanied in its later stages by a temporary increase in the concentration of the constituents of the milk.

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CXXXVII. INSULIN AND THE BLOOD-FAT.

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(*Received October 10th, 1925.*)

In the series of experiments here recorded two dogs were used, their fasting blood-fat being observed for at least one month before the injections of insulin were begun. These preliminary observations were to ascertain the range of normal variation that could exist in these animals consistent with good nutritional condition, so that, knowing this range, it would be possible to evaluate correctly any unusual blood-fat values that might occur. Shortly after the commencement of these observations, it was found that one of the animals, Dog II, was pregnant, so that for some time she was an unsuitable subject for the injection of insulin. Her fasting blood-fat was, however, observed concurrently with that of the other dog, so that an opportunity was available for comparing the blood-fat of pregnancy with that of a normal animal of the same sex under similar dietetic conditions. The fasting blood-fat values for Dogs I and II are given in Table I.

Table I. *Fasting blood-sugar, total fat, and cholesterol.*

	Blood-sugar mg. %		Total fat mg. %		Cholesterol mg. %	
Date	Dog I	Dog II	Dog I	Dog II	Dog I	Dog II
18. ii. 25	110	99	392	489	218	219
24. ii. 25	98	—	403	372	—	—
5. iii. 25	—	132	474	518	226	231
10. iii. 25	—	119	530	530	231	211
11. iii. 25	—	—	815	1056	—	269
13. iii. 25	—	126	656	1056	288	308
17 iii. 25	147	121	471	764	256	256
19. iii. 25	—	131	582	823	228	255
20. iii. 25	103	103	719	808	256	245
21. iii. 25	118	118	675	872	231	260
23. iii. 25	131	101	622	1035	324	350
24. iii. 25	129	86	760	930	306	308
25. iii. 25	124	114	323	427	253	285
26. iii. 25	Dog II whelped					
13. iv. 25	135	116	658	616	272	260
14. iv. 25	—	—	483	602	239	225
15. iv. 25	115	108	468	687	240	270
17. iv. 25	123	110	420	414	289	265
18. iv. 25	117	110	503	531	263	241

The values included under the term "total fat" include all the fatty acid present in the blood, no matter from what source, along with the cholesterol, as determined by Bloor's nephelometric method [1914]. The cholesterol was estimated by the colorimetric method of the same author [1916], blood-sugar by the Folin-Wu technique [1919]. It will be noted that the total fats of Dog II were considerably higher than those of Dog I until the termination of pregnancy, but became more nearly comparable with those of Dog I after parturition. The increase was most marked in the total fat, whilst the cholesterol, although generally slightly higher, did not show such a marked increase. In both cases, however, there were considerable fluctuations in the blood-fat level. The changes observed show a striking similarity to the changes noted in a case of xanthomatosis complicated by diabetes insipidus [Turner, Davidson and White, 1925]. In that case, however, the fluctuations were much more extensive, a great exaggeration of what are probably normal variations. Oshima [1907] found from ultramicroscopic evidence an increase of fat in the blood of the pregnant guinea-pig, whilst Coope and Mottram [1914] found in late pregnancy and early lactation the liver-fat in the rabbit increased, and Dixon and Marshall [1924] showed that the pituitary was more active in the later stages of pregnancy, a series of observations that fall into line with the later findings of Coope and Chamberlain [1925] that pituitrin caused an increase in the fat of the liver, and with the definite signs that can be observed histologically in the gland itself [Daunois and Mulon, 1903].

To return now, to the experiments proper: they may be divided into four groups: (1) two experiments in which crude pancreatic extract was used, (2) five experiments in which insulin (Allen and Hanbury's) was injected subcutaneously, (3) two experiments in which 10 % glucose was given alone, and (4) six experiments in which insulin was given along with carbohydrate. No observations were made of the respiratory metabolism during these experiments, since the taking of blood, in the amounts required, would have vitiated the results.

Previously to determining the action of insulin on the fasting blood, experiments were carried out to see whether the blood-fat underwent any important change during the period the animal would be under observation, namely, during the four hours after a fast of 18 hours' duration. The changes observed were so slight as to be negligible.

The crude pancreatic extract used in the two following experiments was prepared in the laboratory, and had been proved to contain insulin by the marked lowering of the blood-sugar that ensued in a rabbit after its injection. The preparation was simply the precipitate of the pancreatic extract from 93 % alcohol, kept *in vacuo* without further purification. 0·4 g. of this, dissolved in 10 cc. of distilled water, was injected into each dog. The results are given in Table II.

The response of Dog I to the crude extract was irregular as far as the total fat was concerned, but the average total fat for the six hours of the experiment was 10 % higher than the fasting level, despite two readings below the primary level. The cholesterol in this case showed a steady and prolonged fall. A similar fall was noted in the second and pregnant dog.

The total fat in this latter animal, however, fell and remained considerably lower than the fasting value throughout the rest of the experiment. The blood-fat of this animal, nevertheless, was always higher than that of the first dog during the day on which these experiments were performed. In the experiments that followed those given above, in which commercial insulin was used, the changes in the cholesterol level were never of the extent found in Table II.

Table II. *Crude pancreatic extract and the blood-fats.*

Time after the injection of 0·4 g. comm. insulin	Blood-sugar mg. %		Total fat mg. %		Cholesterol mg. %	
Hours	Dog I	Dog II*	Dog I	Dog II	Dog I	Dog II
0·0	124	126	323	1056	252	308
0·5	94	106	382	812	252	260
1·0	93	—	255	—	241	—
1·5	99	—	468	—	238	—
2·0	91	104	243	754	227	243
2·5	93	—	489	—	218	—
3·0	—	104	—	778	—	218
3·5	109	—	410	—	165	—
4·0	103	—	363	—	170	—
4·5	112	121	446	783	164	—
5·5	110	—	309	—	160	—
6·0	108	—	302	—	163	—

* Dog II was pregnant at this time.

(Reckoned per kg. of body weight Dog I received 34 mg. and Dog II 37 mg. per kg.)

In all the experiments in which insulin was given alone there was a rise in the blood-fat, a rise affecting the total fat much more than it affected the cholesterol, and bearing no relation to the extent of fall in the blood-sugar, or to the time of onset of the fall. This absence of relation between the blood-sugar and the blood-fat is not a unique observation. Raper and Smith [1925] found that the fat migrated from the liver only in the extreme stages of insulin action. Similarly, McCrudden and Sargent [1918] found that in various pathological conditions the blood-cholesterol varied independently of the blood-sugar. Table III shows the results (in mg. %) obtained with insulin.

Table III. *The effect of insulin on the blood-fats, etc.*

Exp. No.	Insulin in units per kg.	Insulin in units Total		Time in hours after the injection of insulin 0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0
1	1·67	20	Total fat	656	824	—	798	—	—	789	—	—	770	—
			Cholesterol	288	319	—	264	—	—	241	—	—	—	—
2	0·83	10	Sugar	132	102	74	—	59	—	56	66	67	63	—
			Total fat	592	838	818	—	818	—	701	735	709	661	—
			Cholesterol	265	252	255	—	250	—	238	252	237	244	—
3	0·83	10	Sugar	115	63	53	—	60	72	62	64	66	93	—
			Total fat	468	671	543	—	570	634	423	449	709	712	—
			Cholesterol	240	229	241	—	249	224	227	213	234	251	—
4	0·60	5	Sugar	102	38	32	30	40	—	42	52	77	89	—
			Total fat	453	356	302	333	470	—	405	593	560	616	—
			Cholesterol	270	263	—	252	266	—	247	266	265	267	—
5	0·42	5	Sugar	133	121	94	81	66	—	68	77	80	86	—
			Total fat	438	447	501	489	431	—	508	480	681	698	—
			Cholesterol	227	215	236	223	214	—	213	222	199	219	—

In all these there was a definite rise in the blood-fat, the rise in the first two being immediate and prolonged, while in the third there was a preliminary rise succeeded by a fall to a value below the fasting level, and followed by a rise to an even higher level than was attained in the first stage of the action of the insulin, recalling the irregular rise obtained with the crude pancreatic extract in Dog I. The reasons for these variations are not evident. In the last two cases the rise in total fat was much later but still definite. The blood-sugar in all cases showed the usual response to insulin. The changes in cholesterol were not regular, a considerable fall being noted in the first experiment of the series, a fall not so extensive in the second and third experiments, whilst the variations in the fourth and fifth experiments all lay within the error of the method.

Table IV gives the results obtained after the administration of 100 cc. of 10 % glucose solution *per os*.

Table IV. *Effect on the blood-fat of* 100 *cc.* 10 % *glucose* per os.
0·85 *g. per kg.* (*Mg.* %.)

Exp. No.		Time in hours after the ingestion of glucose										
		0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0
1	Sugar	112	191	178	105	98	—	104	—	111	133	125
	Total fat	641	566	589	526	661	—	622	—	640	695	617
	Cholesterol	261	240	245	231	216	—	227	—	221	213	236
2	Sugar	117	149	166	114	89	—	108	—	105	—	—
	Total fat	597	570	551	528	474	—	513	—	629	—	—
	Cholesterol	278	266	239	232	216	—	241	—	230	—	—

The ingestion of glucose was accompanied by a fall in the total fat affecting chiefly the cholesterol fraction, while the secondary increase in total fat was in large measure due to an increase in the fatty acid fraction of the blood-fat. In an experiment in which glucose was given subcutaneously the preliminary drop in cholesterol was not so marked, but the total fat rose, the increase occurring in two stages. This change in the curve was in all probability due to the different rate of absorption of the glucose when given by this channel.

The results obtained from the administration of insulin and glucose at different intervals are summarised in Table V.

In these experiments the action of insulin on the blood-sugar was never abolished, although it might be delayed by the absorption of the glucose administered. In all but the first experiment there was a rise in the total fat, the extent and time of onset of which varied considerably, being but slightly marked in the fourth experiment of the series. No relation could be observed between the different curves for sugar, fat or cholesterol during the period of experimentation, nor between the intensity of the hypoglycaemia and the rise of fat that occurred. The changes that occurred in the cholesterol values were not very significant in the majority of the experiments, although, in the first experiment recorded in Table V, in which the dosage of insulin was high and the glucose was given during the stage of clinical hypoglycaemia, there was a 20 % fall in the cholesterol in the second specimen, taken before the glucose was given. No definite explanation can be given for the changes in blood-fat that occurred in this same experiment.

Table V. *The effect of insulin and glucose on the blood-fat etc.* (*Mg.* %.)

Exp. No.	Insulin units per kg.	Glucose g. per kg.	Glucose hours after insulin		Time in hours after the injection of insulin 0·0	0·5	1·0	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0	5·5
1	1·7	0·85	1·3	Sugar	123	—	—	—	77	—	—	—	63	62	58	68
				Total fat	591	—	598	491	496	—	—	524	490	510	571	591
				Cholesterol	286	—	229	273	255	—	—	225	234	231	269	—
2	0·85	0·85	1·0	Sugar	123	119	89	44	47	—	84	102	115	119	—	—
				Total fat	505	434	496	554	593	—	523	597	566	546	—	—
				Cholesterol	252	241	231	255	230	—	238	227	233	228	—	—
3	0·85	1·7	0·0	Sugar	132	105	71	63	32	59	55	—	66	60	—	—
		0·85	2·0	Total fat	383	362	355	363	375	351	496	—	431	400	—	—
				Cholesterol	—	227	223	233	211	237	219	—	221	—	—	—
4	0·60	2·38	0·0	Sugar	108	144	98	66	43	—	41	59	73	77	—	—
				Total fat	438	467	380	334	418	—	356	358	368	318	—	—
				Cholesterol	233	236	252	221	226	—	196	220	226	244	—	—
5	0·42	1·7	0·0	Sugar	132	141	108	82	74	87	58	69	78	—	—	—
		0·85	1·0	Total fat	414	363	366	403	468	396	681	592	492	—	—	—
				Cholesterol	236	226	224	216	211	212	224	209	216	—	—	—
6	0·42	0·85	0·0	Sugar	126	152	104	66	62	—	70	77	74	82	—	—
				Total fat	266	342	320	251	347	—	283	297	350	353	—	—
				Cholesterol	190	198	195	201	181	—	183	197	177	197	—	—

Discussion.

The results given in Tables II and III extend to the normal unanaesthetised dog the findings of Raper and Smith [1925] that insulin hypoglycaemia is associated with an increase in the blood-fat. It is known that in cases of diabetic lipaemia insulin may bring about a marked fall in the blood-fat. This, however, may be simply the result of the improved carbohydrate metabolism consequent on the administration of insulin and not so much a direct action. The findings recorded, however, only apply to the normal non-diabetic animal. Whilst dogs were the only subjects used in these experiments, in an experiment in which the writer was the subject it was found that insulin did not lower the blood-fat, at least during the stage of its action on the blood-sugar. There was a slight rise in the blood-fat just outside the limits of error of the method. The giving of carbohydrate along with the insulin or afterwards, did not markedly alter the action of insulin on the blood-fat. Conversely, the injection of insulin does not modify the blood-fat curve obtained after a meal containing fat and oatmeal [Hartmann, 1924]. The only experiment in which insulin *per se* was found in this series to cause a definite and prolonged fall in the blood-fat was one in which the animal was pregnant and crude extract was used. These two factors sufficiently complicated the problem to make any explanation doubtful. Whether this transport of fat from the liver to the muscles is a specific action of insulin or the result of the increased utilisation of carbohydrate cannot be definitely stated from the evidence at present available. Observations on the respiratory metabolism do not tend to support the hypothesis of a direct action of insulin on fat, at least, as far as the ultimate oxidation of fat is concerned. An R.Q. above unity after the administration of insulin has been reported by Macleod [1924]. This was considered to be evidence of the conversion of carbohydrate into fat. Later experiments, however, have failed to confirm this. Further, no evidence has been brought forward to show that insulin causes an increased formation of fat apart from the increase that may be shown to occur as a result of increased transport of fat from the liver. The action of insulin on the blood-cholesterol was not marked except in those cases where crude insulin was used and where the decrease was probably partly, at least, due to the presence of impurities. With purer preparations the changes were less marked and indeed were in several cases within the error of the method. Certainly, when large doses were used, there was a definite fall in the blood-cholesterol. Nitzescu, Popescu-Inotesti and Cadariu [1924] report that insulin has no effect on the cholesterol content of the blood of the normal dog, but one cannot compare exactly the dosages employed in their experiments with those employed above.

Conclusions.

1. When insulin causes a marked hypoglycaemia in the normal dog this is, as a rule, accompanied by an increase in the blood-fat, an increase affecting the fatty acid more than the cholesterol, the extent and time of onset of which vary.
2. The administration of glucose along with insulin, or later, does not seem to affect the changes in blood-fat brought about by insulin.
3. The effect of insulin on the blood-cholesterol was, in the doses used in these experiments, not constant.

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CXXXVIII. THE CONTROLLING INFLUENCE OF CARBON DIOXIDE.

V. A QUANTITATIVE STUDY OF THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY CELLS OF THE HIGHER PLANTS IN RELATION TO CONCENTRATION OF OXYGEN AND CARBON DIOXIDE.

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Introduction.

It has long been known that ethyl alcohol appears as a product of metabolism when many higher plants (including apples) are kept in atmospheres without oxygen [Lechartier and Bellamy, 1872; Pasteur, 1872; Pfeffer, 1900]. More recently an enzyme system essentially similar to the zymase of yeast has been isolated from the tissues of higher plants (including apples) [Palladin and Kostytscheff, 1906; Stoklasa, Ernest, and Chockensky, 1907, 1 and 2], and it is now generally accepted that in the usual respiration of glucose by higher plants this zymase system co-operates with an oxidation enzyme system to produce carbon dioxide. So long as the oxidation system maintains its normal activity the zymase activity of cells will not be demonstrable, but when the oxidation system is inactivated, as through the absence of the component oxygen under anaerobic conditions, zymase activity becomes evident by the accumulation of ethyl alcohol as in the zymasic metabolism of yeast cells. Further, in the usual metabolism of yeast small quantities of acetaldehyde are always produced [Fernbach and Schoen, 1920; Harden, 1923]; and small quantities of acetaldehyde have been detected as accumulating, together with ethyl alcohol, in poplar flowers placed under anaerobic conditions [Kostytscheff, Hübbenet, and Scheloumoff, 1913] and also when a maceration of germinating pea seeds acts upon glucose solution under anaerobic conditions [Neuberg and Gottschalk, 1924]. In this paper the appearance of acetaldehyde, in addition to ethyl alcohol and carbon dioxide, amongst the end products of the zymasic metabolism of apples under anaerobic conditions is recorded. Neuberg and his co-workers by following quantitatively the production of ethyl alcohol and acetaldehyde in the metabolism of yeast under different conditions, have brought strong evidence to support the original contention of Kostytscheff that acetaldehyde is the immediate precursor of ethyl alcohol in alcoholic fermentation [see Fernbach and Schoen, 1920]. The author has adapted the technique used by Neuberg to study quantitatively the accumulation with time of ethyl alcohol and acetaldehyde in apples under different conditions. Such studies evidently give a direct measure of the extent to which zymasic metabolism occurs in the tissues of higher plants. Plant physiologists have studied the respiratory metabolism of higher plants mainly by observing the rates of gaseous exchange and by determining the values of the $\frac{\text{Carbon dioxide}}{\text{Oxygen}}$ ratios; by these methods they have shown that carbon dioxide in certain concentrations may retard the rate of production of carbon dioxide by plant tissues [Jodin, 1897; Kidd, 1917]. By determining the acetaldehyde and ethyl alcohol accumulations in apples in different gases and gas mixtures, the interesting discovery can now be recorded that carbon dioxide—above certain concentrations—even in the presence of abundant oxygen inactivates the oxidation system in apple cells without affecting the zymase system: in such concentrations of carbon dioxide, therefore, the metabolism of apple cells is essentially of a zymasic type. For convenience, the term CO_2-zymasis will be used to describe this zymasic metabolism induced by carbon dioxide in the presence of oxygen. Also, we will speak of the zymasic metabolism occurring in the absence of oxygen as anaerobic zymasis. CO_2-zymasis differs from anaerobic zymasis in pure nitrogen or in pure CO_2 by the greater accumulation of acetaldehyde, and the correspondingly smaller accumulation of ethyl alcohol in the former, while the total destruction of sugar appears to be the same initially.

Acetaldehyde is more toxic than ethyl alcohol; to its greater accumulation in atmospheres containing carbon dioxide (above a certain concentration) plus oxygen may probably be attributed the greater toxicity of such gas mixtures compared with pure nitrogen, pure carbon dioxide, or any mixture of nitrogen and carbon dioxide [Kidd and West, 1923]. Certain very severe cases of damage to large quantities of apples, during overseas transport under cold storage, have recently been traced to the toxicity of mixtures of carbon dioxide and oxygen, and it was, in fact, to elucidate this special toxicity and also to determine other causes of premature death of apples which have been attributed to the accumulation of toxic metabolites, that this investigation was commenced[1].

SECTION 1. THE IDENTIFICATION OF ACETALDEHYDE AND ETHYL ALCOHOL AS PRODUCTS OF THE RESPIRATION OF APPLES IN CERTAIN GASEOUS ENVIRONMENTS.

A. (i) *Demonstration of aldehyde production with intact apples.* Aqueous acid solutions of phloroglucinol (1 % phloroglucinol in 6 % sulphuric acid was used in these experiments) speedily combine with aliphatic aldehydes to produce sparingly soluble substances known as phloroglucides, and with excess of phloroglucinol the amount of precipitation is proportional to the amount of aldehyde present (see Appendix). These facts were exploited for the investigation of the apples. Gas mixtures of different composition were passed at the same rates for the same time-period through desiccators, containing equal numbers of apples (Fen Newtowns), placed in a room maintained at 15°. The emerging gas passed through the acid phloroglucinol reagent. The relative production of volatile aldehydes by apples under the different gaseous conditions was gauged by comparing the bulks of the phloroglucide precipitates obtained. With this technique the aldehyde production cannot be of secondary origin due to injury or to high temperatures.

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[1] This work was taken over from the Hon. Mrs Wheldale Onslow, to whom the idea of investigating the acetaldehyde and ethyl alcohol accumulation under anaerobic conditions is due, and I am greatly indebted to her for the close touch she has maintained with the research during its course.

Composition of gas stream	Result
Air (freed from CO_2)	Very slight precipitation
Oxygen (freed from CO_2)	,, ,,
Nitrogen (freed from CO_2 but contains a trace of oxygen)	Copious precipitation
Carbon dioxide 50 % + oxygen 50 %	,, ,,

The identity of these aldehydic metabolic products had to be established by other methods, as phloroglucides are amorphous substances of ill-defined melting points and of uncertain composition. Using the British apple named above, the author repeated and confirmed the experiment of Power and Chesnut [1920], who used the delicate Rimini reaction to show that acetaldehyde emanates from ripe American apples taken from an air store. So the slight precipitation of phloroglucide in the air and oxygen conditions was at least in part due to the presence of acetaldehyde in the living apples.

(ii) *Evidence that acetaldehyde is the only aldehyde produced in Exp. A* (i). The identity of the volatile aldehyde (or aldehydes), accumulating in living apples under the conditions described above, was determined by re-distilling the steam distillate of the tissue, and preparing from the first fraction crystalline derivatives with *p*-nitrophenylhydrazine [for details, see Dakin, 1908] and with dimethylhydroresorcinol [see Neuberg and Reinfurth, 1920].

p-Nitrophenylhydrazones. After one recrystallisation from dilute alcohol, crystals were obtained of melting point 128·5° identical with that of acetaldehyde *p*-nitrophenylhydrazone synthesised in the laboratory. The M.P. was not altered by mixing the two derivatives.

Dimethylhydroresorcides. After one recrystallisation from dilute alcohol, crystals were obtained of melting point 139–140° identical with that of acetaldehyde dimethylhydroresorcide synthesised in the laboratory. The M.P. was not altered by mixing the two derivatives. As these derivatives were so easily purified, and as no other aldehyde was identified in the first or later fractions, it was concluded that acetaldehyde alone caused the phloroglucide precipitations described in A (i).

B. *Demonstration of simultaneous ethyl alcohol production.* The steam distillates obtained as described in A (ii) were freed from acetaldehyde—by combining this substance with *m*-phenylenediamine hydrochloride (see Appendix)—and redistilled, yielding liquids giving no reactions for ketones, but giving the iodoform reaction.

C. *Conclusions.* Air-stored apples contain traces of acetaldehyde and ethyl alcohol. These substances may accumulate in apples when there is a shortage of oxygen, or under the influence of carbon dioxide in the presence of sufficient oxygen. In the following sections the results of quantitative studies on these facts will be given and discussed.

SECTION 2. THE NORMAL ACETALDEHYDE AND ETHYL ALCOHOL CONTENTS OF SOUND APPLES STORED IN AIR.

The figures in the following table represent grams acetaldehyde found in 100 g. fresh weight of tissue of apples stored in air for different periods from October at 1°, 15° and 22°.

Table I. (See Figs. 1, 2, 3, and 4, air value lines.)

Acetaldehyde in apples kept in air.

Month	1°	15°	22°
October (experiment begun)	0·0003	0·0003	0·0003
December	0·0007	0·0005*	0·0003*
February	0·0005*	0·0007	0·0005
July	0·0006	—	—

The details of the analytical procedure will be found in the appendix.

* Colour change from green to yellow was completed. After February all apples at 15° and 22° were damaged by fungi.

Air-stored apples always contain a little acetaldehyde (see Section 1) which does not increase in amount with extension of the storage period: this confirms the results of Müller-Thurgau and Osterwalder [1915]. The average of the results given above (0·0005 g.) is used in the following sections as the air-value of acetaldehyde in apples.

Power and Chesnut [1920] always detected ethyl alcohol in steam distillates from air-stored apples. In the present experiments, estimations made in June and August, on apples stored at 1° from the previous October, gave at both times 0·006 g. ethyl alcohol in 100 g. fresh weight of apple tissue. As acetaldehyde formation has always been found to accompany the formation of ethyl alcohol in apples, and as acetaldehyde does not accumulate during storage, it is concluded that ethyl alcohol does not accumulate, and that 0·006 % may be taken as its average air value in apples. Probably both air values are too high, as aldehydic and alcoholic substances may form to some extent from apple pulp decompositions at high temperatures, and certainly the volatile esters and terpenes always present in apples will, in part, be oxidised to volatile acids in the estimation of ethyl alcohol as acetic acid (see Appendix for technique).

SECTION 3. THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY APPLES UNDER ANAEROBIC CONDITIONS IN AN ATMOSPHERE OF NITROGEN.

Apples were placed in closed desiccators, containing potash, which were first evacuated and then re-filled with nitrogen from a gas cylinder. Gas analysis with a Haldane apparatus showed that no carbon dioxide accumulated and no oxygen leaked in. The desiccators were placed in rooms at the desired experimental temperature (1°, 15°, or 22°), and the ethyl alcohol and acetaldehyde content of the apples at the end of definite time periods was determined (see Appendix). The results are expressed in Table II as g. substance in 100 g. fresh weight of apple tissue.

Table II. (See Figs. 1 and 2.)

Ethyl alcohol and acetaldehyde in apples kept in nitrogen.

Number of days of anaerobiosis	22°		15°		1°	
	Ethyl alcohol	Acet-aldehyde	Ethyl alcohol	Acet-aldehyde	Ethyl alcohol	Acet-aldehyde
0	0·0060	0·0005	0·0060	0·0005	0·0060	0·0005
1	—	0·0030	—	0·0013	—	—
2	0·217	0·0052	—	0·0023	—	—
3	—	0·0051	—	0·0027	—	—
4	—	0·0063 (*d*)	—	0·0030	—	—
5	0·368 (*d*)	—	—	0·0031	—	—
6	—	—	—	0·0041	—	0·0013
7	—	0·0055	—	—	0·096	—
9	—	—	0·396	—	—	—
10	—	—	—	0·0039 (*d*)	—	0·0028
11	0·664 (*D*)	0·0057 (*D*)	—	—	—	—
13	—	—	0·504 (*d*)	0·0037	—	0·0034
14	—	0·0055	—	—	—	—
15	—	—	—	—	0·170	—
17	—	—	—	—	—	—
19	0·699	—	—	—	—	0·0038
21	—	—	0·829	0·0041	—	—
27	—	—	—	—	0·278	—
30	—	—	—	0·0044	—	0·0037
35	0·663	—	—	—	—	—
38	—	—	—	—	0·389 (*d*)	—

(*d*) Protoplasmic disorganisation of superficial cells commences and continues progressively inwards, until:—

(*D*) Protoplasm of all cells of apple tissue is disorganised. Apples are completely dead.

(i) *The acetaldehyde—ethyl alcohol equilibria in anaerobic zymasis at different temperatures.* The data of Table II show that the anaerobic zymasis of apples resembles the usual metabolism of the yeast cell in that small amounts of acetaldehyde and relatively large amounts of ethyl alcohol are afterwards found present. Harden [1923] writes of yeast: "This of course coincides with the occurrence of acetaldehyde as an intermediate product of alcoholic fermentation." The view is adopted here, that in the anaerobic zymasis of apple cells acetaldehyde is the immediate precursor of ethyl alcohol to which it is reduced by the activity of a reductase system. Its accumulation in these experiments, and in the experiments mentioned in the Introduction to this paper on poplar flowers and peas, cannot yet be precisely explained, but may in some way be associated with the attainment by the reductase system of maximal activity under anaerobic conditions. The gradual flattening of the progress curves for acetaldehyde formation (Fig. 2) would then represent the attainment of equilibria, between aldehyde and alcohol, which are reached more rapidly at higher than at lower temperatures.

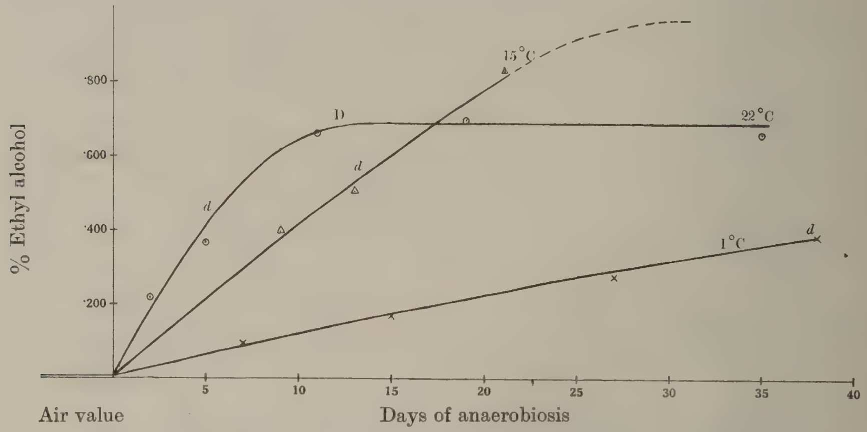

Fig. 1. Production of ethyl alcohol by apples in anaerobiosis (see Table II).

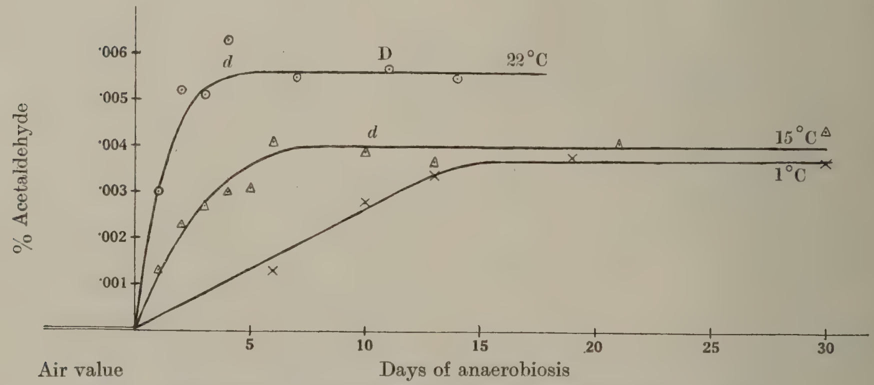

Fig. 2. Production of acetaldehyde by apples in anaerobiosis (see Table II).

It seemed possible at first that the escape of acetaldehyde from apple tissue might account for the flattening of the curves, and to investigate this possibility the phloroglucinol technique (see Appendix) was used to show that the escape of acetaldehyde from aldehyde-laden apples, submitted at 1° for one week to a gas stream from a nitrogen cylinder, is not significant at this temperature. The escape in a closed space will be very much less, and since at 1° the flattening of the aldehyde curve is not an index of the evaporation of acetaldehyde, at all temperatures, some other explanation had to be found. Nor is the upper limit due to toxic arrest, for no universal correlation is observed between the apparent cessation of acetaldehyde accumulation and the disorganisation of the protoplasm, since accumulation at 1° ceased after 15 days, but no injury to cells was visible even after 30 days. The possibility that acetaldehyde is formed from some substance, other than glucose, present in apples only in small quantities gets no support, as different amounts of acetaldehyde are formed at different temperatures.

(ii) *The rates of production of acetaldehyde and ethyl alcohol in anaerobic zymasis.* The initial rates of production are some direct function of the temperature; for example, during the first 48 hours the approximate average rates of production of ethyl alcohol in mg. per hour are, at 22° 4·4, at 15° 1·8, and at 1° 0·6. Retardation of these initial rates and final cessation of acetaldehyde accumulation and of ethyl alcohol production occur more rapidly at the higher temperatures. This retardation in respect of acetaldehyde has just been discussed, and attention will now be focussed on the alcohol progress curves. Retardation and ultimate cessation of alcohol production in nitrogen is correlated with the protoplasmic disorganisation which commences in the superficial cells, and which gradually becomes more profound until all the cells of all the apples under experimental conditions are dead. In this connection it is interesting to note that Lechartier and Bellamy [1872] discovered that sooner or later many fruits—including apples—ceased to produce carbon dioxide under anaerobic conditions. This cessation probably synchronises with the cessation of ethyl alcohol production on the death of the cells. That anaerobic zymasis occurs only in living cells was further shown by the observation that no increase occurred in the acetaldehyde or ethyl alcohol content of apples previously killed by prolonged freezing (one month at − 11°) and subsequently placed at 15° for seven days under anaerobic conditions. After disorganisation has commenced in apples and in other oxidase tissues under anaerobic conditions, browning will occur as a post mortem phenomenon on readmission of oxygen. The death of apple cells under anaerobic conditions is attributed to the ethyl alcohol formed, although the absence of oxygen, and the presence of the more toxic acetaldehyde probably aggravate the effect of the alcohol.

No relation is apparent between aldehyde concentration and rate of alcohol production in anaerobic zymasis; for at 22° the rate becomes markedly retarded after about three days, when the aldehyde concentration is 0·005 %, and by the eleventh day the rate has fallen to one-quarter of the initial value, whereas the aldehyde concentration has only increased to 0·006 %, and it will be shown in Section 5 that concentrations of acetaldehyde less than 0·015 % do not retard the rate of production of ethyl alcohol to any marked extent. Alcohol production in anaerobic zymasis is believed to be a toxic process; the curves for 1° and for 15° were also, without doubt, each tending towards toxic limits which possibly would have been at a higher level at 15° than at 22°, thus explaining the cutting of the curve for 22° by that for 15°.

The dotted part of the curve for 15° is not based on experimental results, but represents approximately what would have been obtained in a more prolonged experiment. At 1° the toxic concentration of ethyl alcohol must be very slowly attained, since no injury was observed until the 38th day, therefore it is not surprising that the steady rate of production of ethyl alcohol did not alter during the period of the experiment.

SECTION 4. THE PRODUCTION OF ETHYL ALCOHOL AND ACETALDEHYDE BY APPLES UNDER THE INFLUENCE OF CARBON DIOXIDE, IN THE PRESENCE OF OXYGEN: CO_2-ZYMASIS.

Carbon dioxide and oxygen, after being mixed in the desired proportions in gasometers of 60 cubic feet capacity, were passed over apples, placed at 15° in desiccators, at a rate sufficient to prevent any appreciable accumulation of carbon dioxide around the apples from their respiration. It has been shown in Section 1 that zymasic metabolism may occur under these conditions, and this has been followed quantitatively by determining the acetaldehyde accumulation after different time-periods in each gas mixture. As the gas around the apples was moving in these experiments, in addition to estimating the aldehyde content of the apples at the conclusion of each time-period by the steam distillation technique, it was necessary to estimate the acetaldehyde escaping during each period. For this purpose the emerging gas was passed through acid phloroglucinol solution, and the weight of aldehyde in the gas current, calculated from the weight of phloroglucide obtained (see Appendix), was added to the residual aldehyde content of the apples as determined by the steam distillation technique. The results are expressed in Table III as g. acetaldehyde in 100 g. fresh weight of apple tissue. The oxygen concentration is in every experiment the percentage complement of the CO_2 concentration.

(i) *CO_2-zymasis.* It will be seen from the table that acetaldehyde is increasing with time in all these cases, whilst in air storage there is no such increase. It will be shown later, in Section 5, that alcohol is also increasing so that we have the metabolism associated hitherto with anaerobic conditions also brought about by sufficient CO_2 in spite of the presence of oxygen. This special effect, which we may call CO_2-zymasis, is probably due to CO_2 inactivating the oxidation system concerned in normal respiration without influencing zymase splitting. Willstätter and Stoll [1918] have shown that the activity of peroxidase systems is diminished to one-half in water saturated with carbon dioxide, though no destruction of enzyme occurs, and their observations are of interest in showing that carbon dioxide has an inhibiting effect on at least one oxidation system. As the apple tissue used never gave p_H values > 4, no connection can exist between the phenomena recorded and the concentration of hydrogen ions, since carbon dioxide will not alter a p_H so much on the acid side of neutrality. Without attempting to analyse the

Table III. (See Fig. 3.)

Acetaldehyde formation in apples by CO_2-zymasis at 15°, in relation to concentration of carbon dioxide.

Days in gas mixture	Experimental Series 1 (Spring)						Experimental Series 2 (Summer)		
	12 % CO_2 I	20 % CO_2 II	30 % CO_2 III	50 % CO_2 IV	70 % CO_2 V	100 % CO_2 VI	50 % CO_2 VII	95 % CO_2 VIII	99 % CO_2 IX
0	0·0005	0·0005	0·0005	0·0005	0·0005	0·0005	0·0005	0·0005	0·0005
1	—	—	—	0·003	0·003	0·003	—	—	—
2	—	—	—	—	0·005	0·004	—	—	—
3	—	—	0·001	0·004	0·004	0·005	0·015	—	—
4	—	—	—	0·009(*A*)	—	—	—	—	—
5	—	—	—	—	0·010(*A*)	—	—	0·036(*d*)	0·006
6	—	—	0·006	—	—	—	0·038(*d*)	—	—
7	—	0·003	—	—	—	—	—	—	—
8	—	0·001	—	—	0·019	—	—	0·052	0·015(*d*)
9	—	—	0·012(*d*) (*A*)	—	—	—	—	—	—
11	—	—	—	·028(*d*)	0·030(*d*)	0·006	0·058(*A*)	—	—
12	—	—	0·033	—	—	—	—	0·057(*A*)	0·014
15	—	—	—	—	—	—	0·055	0·054(*D*)	0·025
16	—	0·015(*d*)	—	—	—	—	—	—	—
17	—	0·012	—	—	—	—	—	—	—
20	—	—	—	—	—	0·006(*d*)	—	—	—
21	0·001	—	—	—	—	—	—	—	—
24	—	—	—	—	—	—	0·057(*D*)	—	—
29	0·002	—	—	—	—	—	—	—	—
50	0·012(*d*)	—	—	—	—	—	—	—	—

(*A*) Browning of cells near the apple core noticed (Brownheart [Kidd and West, 1923]).

(*d*) Protoplasmic disorganisation (with browning, when oxygen is present) of superficial cells commences and continues progressively inwards (Deep scald [Kidd and West, 1923]), until:—

(*D*) Protoplasm of all cells of the apple is completely disorganised (apple is completely brown, when oxygen is present).

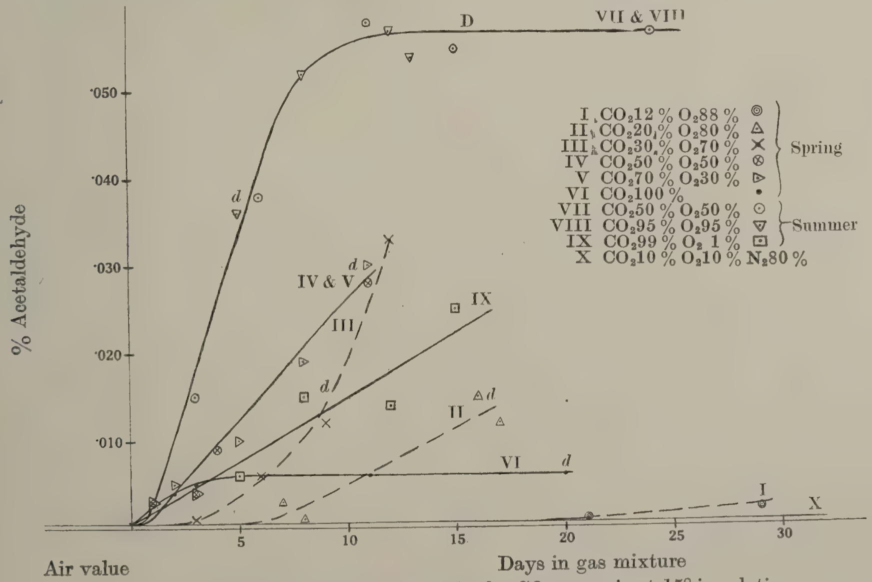

Fig. 3. Acetaldehyde formation in apples by CO_2-zymasis at 15° in relation to concentration of CO_2 (see Table III).

cause of this action of carbon dioxide, we will consider certain aspects of the effects produced, and the first point is that there seems to be a lower limit of concentration of CO_2 below which CO_2-zymasis is not produced. For these "Newtown Wonder" apples this is placed tentatively at 10 % CO_2, as no increase was observed in the acetaldehyde content of apples which had been stored at 8° for six months in a gas mixture of 10 % CO_2, 10 % oxygen, 80 % nitrogen, but in this connection we shall see later in this section that future research may necessitate a change in our views. The second point is that the co-operation of oxygen is needed for CO_2-zymasis to occur, as anaerobic zymasis, of the type described in Section 3, occurs in pure CO_2. Anaerobic experiments were set up precisely as described in the last section, but using pure CO_2 instead of pure N_2. With mixtures of CO_2 and O_2 between these limits the effect is well marked but the precise nature of the relation is complicated by the senescent state of the apples and will be discussed later (see p. 937). The third point is that much more acetaldehyde accumulates in the tissues under CO_2-zymasis than in anaerobic zymasis. This will be considered further in Section 5 where the alcohol production of the two states is compared, but we note here the difference between curves IV, V, VII and VIII in Fig. 3, and the anaerobic curves whether the gas environment is pure nitrogen (Fig. 2) or pure carbon dioxide (Fig. 3, curve VI). When the external oxygen concentration is between zero and 5 %, observed effects are intermediate between CO_2-zymasis and anaerobic zymasis (Fig. 3, curve IX), and these are to be expected, as evidently it is the concentration of oxygen in the intercellular spaces that determines which type of zymasis will occur in different localities in a given apple, and these concentrations will probably, in a bulky tissue such as an apple, be smaller in the localities furthest removed from the lenticels and other points at which oxygen enters from the outside atmosphere.

(ii) *Progress curves for acetaldehyde formation in CO_2-zymasis.* Curve VII (which cannot, on the results obtained, be distinguished from curve VIII) represents a typical progress curve for acetaldehyde formation in CO_2-zymasis, and curve IV (which cannot, on the results obtained, be distinguished from curve V) represents the initial phase of acetaldehyde accumulation under similar CO_2 concentrations, but at an earlier stage in the storage season when the apples are less senescent. By comparing curves VII and VIII or curves IV and V we see that at a given stage of the progressive senescence during storing, provided that more than 5 % oxygen is present, the rate and extent of CO_2-zymasis, and the incidence of the (*d*) condition of superficial browning appear to be the same in all concentrations of CO_2 greater than 50 %. By comparing curve IV with curve VII we see that for a given concentration of CO_2 (50 % in this case), the rate of CO_2-zymasis must increase as the senescence of apples advances in the later stages of the storage season.

The oxidase browning mentioned above is caused by the disorganisation of the protoplasm in the superficial cells in an atmosphere containing oxygen, and the flattening of the progress curves for acetaldehyde production in CO_2-zymasis proceeds hand in hand with the increasing profundity of tissue-browning, in striking contrast with the flattening of the aldehyde curves in anaerobic zymasis (Fig. 3, curve VI and curves in Fig. 2) where cessation of acetaldehyde accumulation occurs before the disorganisation of protoplasm commences. Further, progressive injury and death of cells from the surface inwards (Deep scald [Kidd and West, 1923]) occur more rapidly in CO_2-zymasis than in anaerobic zymasis, and we notice in CO_2-zymasis a type of injury (A), described as browning of cells near the apple core, which does not occur in anaerobic zymasis (Brownheart [Kidd and West, 1923]). Athough Brownheart may occur under the influence of CO_2 without acetaldehyde formation [Kidd and West, 1923], the author definitely attributes the more rapid death of superficial cells in CO_2-zymasis to the greater quantities of the toxic acetaldehyde produced. These matters will be further discussed in the next section; here we note that whereas concentrations of 0·004–0·006 % acetaldehyde, found under anaerobic conditions, are not injurious to apples, concentrations of 0·03–0·05 % retard CO_2-zymasis and finally cause its cessation at the death of the tissue.

(iii) *CO_2-zymasis in relation to the concentration of CO_2 and the resistance of individual apples.* We have seen that early in the storage season the reaction of a given apple to CO_2, as shown by the rate of aldehyde accumulation, must be different from its reaction at a later period. This difference is now attributed to the diminishing resistance to CO_2 with the increasing senescence of the apple. We will consider the behaviour of apples in concentrations of CO_2 between 10 and 50 % and it will shortly be obvious that, at a given period in the storage season, the apples used in these experiments must have been in different senescent states as judged by their varying resistances to such concentrations.

Table IV. *Comparison of the average acetaldehyde contents of non-browned apples and browned apples in samples previously identically treated.*

% CO_2	Days in gas	Number of non-browned apples	% acetaldehyde in non-browned apples	Number of browned apples	% acetaldehyde in browned apples
50–75	11	—	—	6	0·030
30	9	2	0·0004	4	0·018
30	12	—	—	6	0·033
20	16	3	0·0003	3	0·036
20	17	2	0·0002	4	0·023

It has already been shown that as a result of acetaldehyde production in CO_2-zymasis the surfaces of apples become brown, and it was noticed, after certain time-periods in concentrations of CO_2 of 20 and 30 %, that whereas some of the apples in the experimental samples of six appeared to be brown, others still appeared to be healthy. In Table IV are recorded the results of separate analyses of such browned and non-browned classes in samples of apples previously identically treated. It was in some cases difficult to judge whether apples were brown or non-brown; but when an apple, on cutting, gave the characteristic smell associated with zymasis it was included in the browned class, although it is very probable that in some instances zymasis had been of shorter duration than in the obviously browned apples. Separate analyses of individual apples in samples have not yet been made.

These figures prove that the apple population used in all the experiments described in this paper must be considered as mixed, since some of the apples under 30 % CO_2 for nine days had accumulated acetaldehyde whereas in at least two apples no CO_2-zymasis had occurred, and other samples under 20 % CO_2 for 16 and 17 days gave even more striking figures. Indeed, assuming a linear relation, calculation shows that some apples will in nine days have accumulated 0·02 % acetaldehyde in 20 % CO_2, and we have just noticed that in other apples no zymasis occurred during this period in 30 % CO_2; however, it will be made clear later that a given apple responding to 20 % CO_2 will respond to 30 % CO_2 even more rapidly. Winkler [1923] has shown that apples picked from different sides of the same tree subsequently remain healthy for different lengths of time in air storage at low temperature, and there are many other observations to show that mixed effects are to be expected unless special precautions, as to aspect, age of tree, etc., are taken in picking apples and no such precautions were taken in picking the "Newtown Wonder" apples used in these experiments. These mixed effects in relation to CO_2 result from the differences in resistance to CO_2 shown by individual apples which have, in equal periods of storage, reached different stages of senescence owing, at least in part, to the differences in their orchard histories. Taking the time required for CO_2-zymasis to commence in an apple as a direct measure of its resistance to a given concentration of carbon dioxide, it seems probable from Table IV that the scatter of the resistances of individual apples in a given sample decreases as the incident CO_2 concentration increases. Thus for 20 % CO_2 the figures in Table IV indicate a wide scatter of resistances, as some apples must have commenced zymasis at an early stage whereas others show no zymasis even after 17 days. We have not sufficient data to compare the effects of 30 % CO_2 with 20 % CO_2, but there is evidently a fairly wide scatter of resistances to 30 % CO_2 and in concentrations of 75 % CO_2 no differences in resistance have been detected by the methods of analysis employed. With more accurate observations it seems certain that a small scatter of resistances would be detected for all concentrations, the scatter always becoming less as the CO_2 concentration is increased.

From consideration of the average accumulation of acetaldehyde in CO_2-zymasis in given samples in different concentrations of CO_2 as shown in Fig. 4, it is believed that in all concentrations of CO_2 the average initial rate of zymasis increases at first slowly and then more rapidly until the toxic effect of the acetaldehyde comes into play in all cases, causing retardation and finally cessation of production as shown in curves VII and VIII. Thus progress curves I, II and III would have been definitely sigmoid in shape if observations had been made after longer times with 12 % CO_2, 20 % CO_2 and 30 % CO_2. No figures for the early periods in concentrations of CO_2 > 50 % are available; progress curves have, however, in conformity with the ideas just expressed, been drawn in slightly sigmoid form; but no attempt has been made to indicate the beliefs that with more accurate measurements, firstly, curve V for 70 % CO_2 in the spring would have been slightly nearer the y axis than curve IV for 50 %, and curve VIII for 95 % CO_2 in the summer would have been slightly nearer the y axis than curve VII, and secondly, with the higher concentrations of CO_2 the more nearly will progress curves approximate to a logarithmic form. Comparing curves I, II, III and IV, we see that as the concentration of CO_2 increases from 12 % to 50 % the forms of progress curves change from sigmoid with longer bases to sigmoid with shorter bases, thus gradually approaching a logarithmic form[1].

Evidence will now be presented that at a given time in the storage season, once the resistance of an individual apple has been overcome, CO_2-zymasis will occur in an apple at the same rate and to the same extent independently of the inducing concentration of CO_2. Firstly, there is the obvious evidence of curves IV and V and curves VII and VIII which, for we now see that they are not in theory coincident, must be close and parallel to one another; evidently the resistances of all apples are very speedily overcome under concentrations of $CO_2 > 50$ %, so that raising the concentration of CO_2 from 50 to 95 % does not appreciably alter the average rate or the average extent of zymasis in a sample. Secondly, it will be noticed in Table IV that in one experiment with 30 % CO_2, by chance, six apples of low resistance were used and a similar amount of acetaldehyde had accumulated in them during 12 days as would have been expected under concentrations of CO_2 greater than 50 %.

At least two causes may be important in overcoming the resistance of an individual apple to a given concentration of CO_2; firstly, there is the cumulative effect of CO_2 with time, and secondly, there is the increasing senescence of the apples. Considering the second factor, it is evident that, under the experimental conditions employed, the advance of senescence of the apples—which had previously been kept for six months at 1°—must have been accelerated by removal to 15°, and also by the high concentrations of oxygen used in conjunction with the lower concentrations of CO_2. With 12 % CO_2 (curve I), the oxygen concentration surrounding the apples was 88 %, and only slight zymasis had occurred after 30 days, although we see from Table III that during 50 days considerable zymasis had occurred. Evidently there are many points which require further elucidation, for although it has already been reported in paragraph 1 of this section that apples have been kept for six months in a gas mixture of 10 % CO_2, 10 % oxygen, 80 % nitrogen at 8° without showing zymasis (Fig. 3, line X), it seems possible that, with greater oxygen concentrations and also at a higher temperature (15°), the senescent states of apples may so advance that 10 % CO_2 or less might, given time, overcome some of the individual resistances of apples and thus induce zymasis.

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[1] These effects fall into the same schema as the experiments of Henderson Smith [1921] on the killing of Botrytis spores by phenol. In these experiments, however, no attempt has been made to investigate the effect of varying concentrations of CO_2 on the rate of killing of apples; attention has been fixed on the toxic chemical changes intermediate between the commencement of the reaction to CO_2 and the death of the apples.

Section 5. A QUANTITATIVE COMPARISON OF ANAEROBIC ZYMASIS AND CO_2-ZYMASIS.

For anaerobic zymasis we have the data of Table II from experiments carried out in closed desiccators. For comparable experiments on CO_2-zymasis, apples were placed at 1° in closed desiccators containing gas of the initial composition of 50 % CO_2 + 50 % O_2, and after definite time periods their acetaldehyde and ethyl alcohol contents were determined by the steam distillation technique (see Appendix). After the longest period (34 days) the gas composition in the desiccators was 78 % CO_2 + 22 % O_2. It has been shown in Section 4 that such an increase in CO_2 concentration does not appreciably influence acetaldehyde production, and therefore probably has no effect on alcohol production. In Table V the analyses of these apples are

Table V. (See Fig. 4.)

Comparison of ethyl alcohol and acetaldehyde production in apples by CO_2-zymasis and anaerobic zymasis.

Days in gas	Gas mixture initially 50 % CO_2 + 50 % O_2 rising to 78 % CO_2 + 22 % O_2		Nitrogen gas	
	Ethyl alcohol	Acetaldehyde	Ethyl alcohol	Acetaldehyde
0	0·0060	0·0005	0·0060	0·0005
3	—	0·010	—	—
5	0·046	—	—	—
6	—	—	—	0·0013
7	—	0·021 (*A*)	0·096	—
8	0·056 (*A*)	—	—	—
10	—	—	—	—
13	—	—	—	0·0028
15	—	0·032 (*d*)	0·170	0·0034
16	0·079 (*d*)	0·037	—	—
19	—	—	—	0·0038
23	0·084	—	—	—
25	—	0·042 (*D*)	—	—
27	—	0·040	0·278	—
28	—	—	—	0·0037
32	—	0·040	—	—
34	0·080 (*D*)	—	—	—
38	—	—	0·389 (*D*)	—

(*A*) Browning of cells near the apple core noticed.

(*d*) Protoplasmic disorganisation (with browning, when oxygen is present) of superficial cells commences and continues progressively inwards, until:—

(*D*) Protoplasm of all cells of the apple is completely disorganised (apple is completely brown, when oxygen is present).

contrasted with data for anaerobic zymasis at 1°. Since acetaldehyde accumulation is of the same order in pure CO_2 (Table III, column V) as in pure nitrogen (Table II), Table V may be taken to illustrate the influence of oxygen on the nature and amounts of the products of CO_2-zymasis. The results are expressed as g. acetaldehyde in 100 g. fresh weight of apple tissue.

(1) *Comparison of the* $\frac{\text{alcohol}}{\text{aldehyde}}$ *ratios after anaerobic zymasis and* CO_2*-zymasis.* It will be seen that though concentrated CO_2 can produce zymasis in the presence of oxygen yet the resulting accumulation of aldehyde and alcohol does not follow the same course as with zymasis in anaerobic conditions. The proportions of alcohol and aldehyde differ greatly in the two cases. A comparison of curves I with curves II in Fig. 4 shows that in CO_2-zymasis, in correlation with the presence of oxygen, considerably more acetaldehyde and correspondingly less ethyl alcohol accumulate than in anaerobic zymasis, and this point is emphasised in the following table in which the ratios of ethyl alcohol to acetaldehyde accumulations at different times in the experiments are given.

Days	Anaerobic zymasis $\frac{\text{alcohol}}{\text{aldehyde}}$	CO_2-zymasis $\frac{\text{alcohol}}{\text{aldehyde}}$
5	65	2·6
10	50	2·0
20	54	2·4

This increased accumulation of acetaldehyde in CO_2-zymasis cannot be attributed to the oxidation of ethyl alcohol by oxygen, as it will be shown in Section 6 that apple tissue does not effect such an oxidation. The author believes that the difference between the two types of zymasis is due to the effect of oxygen on the reductase system controlling the reduction of acetaldehyde to ethyl alcohol, the final step in the zymase cleavage of glucose. Under anaerobic conditions as soon as the reductase system attains maximal activity acetaldehyde accumulation ceases. In CO_2-zymasis the continuous presence of more than 5 % oxygen retards the reduction of acetaldehyde to ethyl alcohol, so acetaldehyde accumulates as long as the zymase system remains active, and the amount of ethyl alcohol formed is correspondingly reduced.

(2) *A comparison of the rates of anaerobic zymasis and* CO_2*-zymasis.* The initial rates of anaerobic and CO_2-zymasis were apparently identical since in the former the percentage accumulation of alcohol + aldehyde was 0·054 % after five days, and in the latter 0·052 %. During the period of the experiment the rate of anaerobic zymasis (as measured by alcohol production) was not retarded to any marked extent, nor was any injury observed until 40 days had passed. It was quite otherwise in CO_2-zymasis, as after five days the rates of formation of both ethyl alcohol and acetaldehyde were retarded progressively with the disorganisation of protoplasm as described in Section 3, paragraph (ii). CO_2-zymasis occurs only in living cells, for, apart from the obvious evidence of the

curves II in Fig. 4, the author, by prolonged freezing, killed apples, then placed them in 50 % CO_2 + 50 % oxygen for seven days and noticed that neither alcohol nor aldehyde was produced. This accelerated death of apple cells in atmospheres of carbon dioxide + oxygen has already, in the last section, been attributed to the increased acetaldehyde accumulation under these conditions.

In conclusion, the influence of acetaldehyde on the rate of production of ethyl alcohol in CO_2-zymasis will be considered. Assuming that low concentrations of acetaldehyde have no retarding effect, we may as a standard for comparison represent the initial constant rate by the figure 100. That in the later stages of zymasis an inverse ratio seems to exist between the aldehyde concentration in the apples, and the rates at which alcohol is produced, is indicated in the following table where these rates, calculated in terms of the standard from the slopes of the tangents drawn at different points on the alcohol progress curve II in Fig. 4, are shown as decreasing regularly to zero as the acetaldehyde concentration increases.

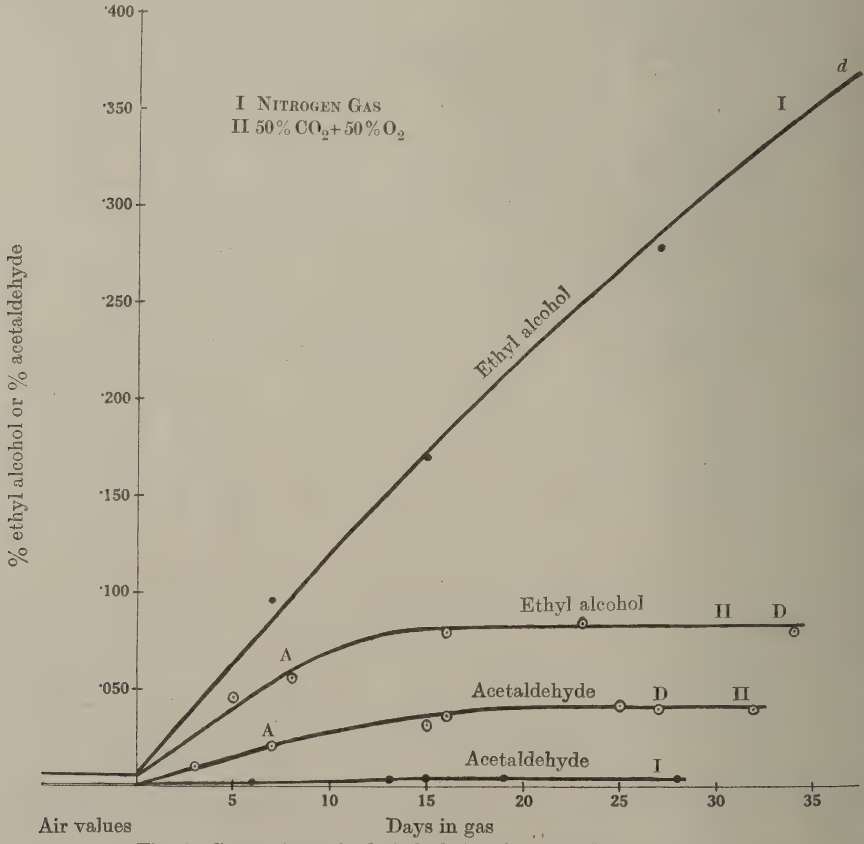

Fig. 4. Comparison of ethyl alcohol and acetaldehyde production at 1° in CO_2-zymasis and in anaerobic zymasis (see Table V).

Acetaldehyde concentration in g. in 100 g. fresh weight of tissue	Relative velocity of alcohol formation in CO_2-zymasis
0·0005 – some value $<$0·017	100 (standard)
0·017	65
0·023 (*A*)	52
0·028	43
0·034 (*d*)	24
0·038	13
0·043 (*D*)	3–0

(*A*), (*d*) and (*D*) as in Table V.

SECTION 6. QUANTITATIVE EXPERIMENTS PROVING THAT APPLE TISSUE DOES NOT OXIDISE ETHYL ALCOHOL OR ACETALDEHYDE.

If, as is generally accepted, the usual oxidation of glucose by higher plants is effected by the co-operation of a zymase and an oxidation system, evidently ethyl alcohol or at least one of its precursors must be substances oxidisable by plant cells. Space does not permit of an adequate discussion here of theories of the biochemistry of sugar oxidation, but mention must be made of Kostytscheff's observation [1909] that ethyl alcohol is not oxidised to carbon dioxide by higher plants, and of the experiments of Ciamician and Ravenna [1918] who showed that crushed spinach leaves, which can effect many interesting oxidations, will not oxidise either ethyl alcohol or acetaldehyde. The following experiments provide further evidence that tissues of higher plants cannot oxidise either ethyl alcohol or acetaldehyde and therefore some precursor of acetaldehyde must be oxidised to produce carbon dioxide in aerobiosis. Apples were kept under anaerobic conditions at 15° for definite periods, when the alcohol and aldehyde accumulations were determined by analysing samples. The remaining apples were analysed after further periods in CO_2-free air, or CO_2-free oxygen.

Days in nitrogen	Subsequent days in air	% ethyl alcohol
9	0	0·396
9	7	0·450
9	14	0·420

We notice that there is no loss of ethyl alcohol either by oxidation or evaporation. Similar experiments were carried out for acetaldehyde: the tabulated results show that there is no loss of acetaldehyde either by oxidation or evaporation. Further as there is no increase in acetaldehyde, ethyl alcohol is not oxidised to acetaldehyde by apple tissue in the presence of oxygen (Section 5, paragraph (i)).

Days in nitrogen	Subsequent days in air	Subsequent days in oxygen	% acetaldehyde
3	0	0	0·0027
3	8	0	0·0025
3	0	6	0·0028
3	0	13	0·0025

SUMMARY.

1. Neither ethyl alcohol nor acetaldehyde accumulates as intermediate or end product of the respiration of apple cells in air; nor can either of them be oxidised by apple tissue.

2. In the absence of oxygen, respiration is of a zymasic type, ethyl alcohol and traces of acetaldehyde accumulating in apple cells. The term anaerobic zymasis is used to describe the effect.

3. Carbon dioxide even in the presence of abundant oxygen may cause the respiration of apple cells to be changed to a zymasic type. The term CO_2-zymasis is used to describe the effect. Whereas CO_2-zymasis commenced nearly immediately in all the apples used in these experiments under concentrations of $CO_2 > 50\ \%$, a wide scatter of resistances to concentrations of CO_2 between 10 and 30 % was noticed.

4. In CO_2-zymasis, associated with the presence of oxygen, the ratio ethyl alcohol/acetaldehyde is 2/1, contrasting strikingly with the ratio 50/1 obtained in anaerobic zymasis.

5. Gas mixtures of carbon dioxide and oxygen may be even more injurious to apples than anaerobic conditions, owing to the toxicity of acetaldehyde being greater than that of ethyl alcohol. The relations of these substances to the so-called physiological diseases of apples will be discussed elsewhere, in a later paper.

The analytical part of this work was carried out in the biochemical laboratory at Cambridge and I am grateful to Sir F. G. Hopkins for the facilities given, and for the interest with which he has followed the research. I express my gratitude to Dr F. F. Blackman for the general supervision he has given during the course of this research, and I also record my thanks to him and to Dr Franklin Kidd for their valuable criticisms on the interpretation and presentation of the results. The physiological part of this research was performed at the Low Temperature Research Station, Cambridge, and I thank Dr Franklin Kidd and Dr Cyril West for placing their experience, on request, at my disposal. To Mr S. G. Crouch, of this Station, I am indebted for assistance in controlling experimental conditions. I thank the Food Investigation Board for a personal grant, and for defraying all expenses incurred during the research.

APPENDIX.

MATERIAL AND METHODS OF QUANTITATIVE ANALYSIS.

Material. "Newtown Wonder" apples, grown in the locality of Canterbury on a gravel soil, were used in all the quantitative experiments which have been described. The apples were picked regardless of their aspect, so it is not surprising that in some respects they behaved as a mixed population (Section 4, paragraph (iii)). On arrival at the Low Temperature Research Station, Cambridge—two days after picking—the apples were wrapped in papers impregnated with mineral oil and placed, in air, in a room kept at a constant temperature of 1°, and were unwrapped and removed just before each experiment started. With the apparatus available, six apples formed the most convenient sample, and this number was used in determining every figure given in the tables.

Methods of quantitative analysis.

(1) *Estimation of the acetaldehyde and the ethyl alcohol content of apples.* A steam distillation technique was used to expel the acetaldehyde and the ethyl alcohol from apples immediately after their removal from the physiological conditions of the experiment in progress. 1500 cc. of water were placed in a two litre flask, through the stopper of which passed the tapered base of a glass cylinder (of dimensions $48'' \times 2\frac{3}{4}''$) wrapped with cotton wool jackets. The water was boiled vigorously until the walls of the cylinder were very hot, and then the previously weighed apples were each cut into eight pieces and dropped into the cylinder, where they rested on a support of perforated zinc, kept in position by a rubber collar. The cylinder was then immediately connected, by means of a splash trap passing through a stopper of $2\frac{3}{4}''$ diameter, to a Liebig condenser previously connected by means of an adapter with an ice-cooled spiral condenser, the base of which passed through the stopper of a filtering flask of 1 litre capacity to a level below that of 50 cc. of liquid placed in the flask. The aqueous distillate collected in the filtering flask. To prevent loss of aldehyde or alcohol, the outlet of the filtering flask was connected with a second filtering flask by means of a bent glass tube which reached a level, in this second flask, below that of 50 cc. of liquid placed in the flask.

In estimating the acetaldehyde content of apples, 50 cc. of sodium hydrogen sulphite—of strength at least double that necessary for combination with the anticipated amount of acetaldehyde—were placed in each of the receiving flasks, and in a control flask. All the flasks were cooled by ice. Experience showed, first, that acetaldehyde only rarely escaped to the second flask, and then only in traces, and second, that after two hours' steam distillation all significant acetaldehyde was expelled from apple pulp. The amount of acetaldehyde which distilled was estimated by titrating the sodium hydrogen sulphite in the receiving and control flasks against standard iodine, using starch solution as indicator. The method, in all its essentials, is that of Ripper [1900].

In estimating the ethyl alcohol content of apples, 50 cc. of water were placed in each of the ice-cooled receiving flasks to prevent any alcohol from escaping. Three hours' steam distillation was necessary to expel all significant ethyl alcohol from apple pulp. The aqueous distillate was made up to a volume of 1 litre, and the alcohol content of each 500 cc. part was simultaneously determined by following the technique described by Neuberg and Hirsch [1919]; each part was first freed from acetaldehyde by adding *m*-phenylenediamine hydrochloride, allowing to stand for half-an-hour, and then gently boiling under a reflux condenser for one hour. After cooling, the resulting brown green fluorescent solution, plus the washings from the reflux condenser, was made just alkaline with caustic soda using solid phenolphthalein as an indicator, and was subsequently distilled nearly to dryness *in vacuo*. The alcoholic distillate was collected in a filtering flask to the outlet of which was connected a bent glass tube dipping under 50 cc. of concentrated sulphuric acid placed in a second filtering flask, which was also connected by pressure tubing with a vacuum water pump. Both flasks were cooled with ice. The concentrated sulphuric acid absorbed any alcohol escaping from the main distillate by forming, for the most part, ethyl hydrogen sulphate [see Dox and Lamb, 1916]. The distillate in the first flask and 20 g. of powdered potassium dichromate were then placed in a Claisen flask connected with a Liebig condenser, and to them were slowly added through a dropping funnel the acid contents of the second flask. Complete oxidation of ethyl alcohol to acetic acid occurred in half an hour at room temperature under these conditions, and by subsequent distillation—with frequent additions of water to the Claisen flask—all the acetic acid was collected. Titration against standard caustic soda gave values from which the alcohol content of the original steam distillate was calculated.

(2) *The estimation of the acetaldehyde escape from apples.* In the experiments in which gas currents were used, the escape of acetaldehyde was gauged by the phloroglucinol method referred to in Section 1 of this paper. Councler [1896] described the phloroglucides, formed by the condensation of the lower aliphatic aldehydes with phloroglucinol in acid solution, as amorphous compounds only sparingly soluble in the medium in which they are formed. Gravimetric estimations of formaldehyde [Clowes, 1899] and furfuraldehyde [Kröber, 1901] are based on the insolubility of their phloroglucides. Clowes does not discuss amounts of formaldehyde less than 0·05 g., and Kröber specifically limits the reliability of his method to amounts between 0·03 and 0·3 g. The author endeavoured, by applying Councler's discovery, to evolve a method for estimating acetaldehyde, in amounts less than 0·03 g., gravimetrically as acetaldehyde phloroglucide. 10 cc. of an acetaldehyde solution (simultaneously standardised by the Ripper method) were added to 40 cc. of a solution of 1 % phloroglucinol dissolved in 6 % (by volume) aqueous sulphuric acid (it was essential in the experiments on apples to use a non-volatile acid). The phloroglucide quickly appears as a white precipitate, which on standing turns yellow. After 24 hours the phloroglucide was collected by filtration through a weighed Gooch crucible, washed with 100 cc. distilled water, and then dried for four hours in a steam-bath in which the oxygen tension was reduced by evacuation with a water-pump, as phloroglucides absorb oxygen rapidly at high temperatures. The crucible was then placed in a weighed covered vessel, cooled in a desiccator and weighed. The

following results on plotting lie approximately on a straight line, which may be used for determining acetaldehyde in quantities greater than 0·015 g.

g. acetaldehyde	g. phloroglucide
0·016	0·017
0·019	0·054
0·033	0·158
0·042	0·247

Although the method for several reasons (the discussion of which is postponed) is not recommended for work requiring a high degree of accuracy it has proved most useful in the experiments of comparison described in this paper. The conditions employed in obtaining the figures given above were, as far as possible, repeated when estimating the acetaldehyde escape from apples.

Each gas stream on emerging from the desiccators containing apples passed through 40 cc. of a solution of 10 % phloroglucinol in 6 % (by volume) sulphuric acid, to which were added 10 cc. of distilled water to make up the volume of the precipitating medium to 50 cc. After the apples had been removed from the desiccators for analysis by the steam distillation technique, the phloroglucide precipitates were treated precisely as described above, and from their weights the acetaldehyde escape (if greater than 0·015 g.) was obtained by referring to the line plotted from the results given above.

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CXXXIX. OBSERVATIONS ON THE EFFECT OF VARIOUS CARBOHYDRATES ON THE KETOSIS OF STARVATION IN HUMAN SUBJECTS.

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It is well known that in man after a relatively short period of starvation, or during a period of diet very low or entirely deficient in carbohydrate, a considerable ketosis appears. The time of onset of a positive nitroprusside reaction in the urine depends upon the composition of the diet prior to the commencement of starvation. A previous diet high in carbohydrate will postpone the ketosis whilst one high in fat and protein and low in carbohydrate will bring it on much more rapidly. Since during starvation the organism is thrown back upon its own tissues, it follows that what is going to happen will depend upon the composition of these tissues. Hence in fat subjects we expect a more rapid onset of ketosis than in thin subjects. This is well illustrated in the case of a starving fat woman investigated by Folin and Denis [1915]. This woman excreted after one day's fasting 270 mg. of acetoacetic acid and in succeeding days very large quantities of acetoacetic and hydroxybutyric acids. The professional faster Succi, who, according to Brugsch's description [1905], seems to have been a man of average build, produced much smaller quantities of the ketonic substances than the fat woman, even after very much longer periods of starvation. In general a man of medium build will not show a positive nitroprusside reaction in the urine in less than 30 hours of complete starvation (no food or drink). In our experience of a considerable number of fasts the time varied from 20 hours after a previous diet low in carbohydrate to 36 hours after an average mixed diet.

The cause of the ketosis seems to depend principally on carbohydrate deficiency. Once a certain minimum of carbohydrate is reached further depletion finds expression in ketosis.

As a result of careful observation of the time of onset of a recognisable ketosis in starving normal subjects, we have received the impression that the onset of acetonuria is essentially an acute phenomenon. If the urine of a starving subject be examined after the first 24 hours' starvation at hourly intervals for 5 or 6 hours, and then every 15 minutes, one suddenly reaches a urine giving a marked nitroprusside reaction. Quantitative estimations of ketonic substances in two consecutive urines separated by a 15 minutes' interval, one being negative and the other positive to the nitroprusside test, show a difference of from perhaps 3 or 4 mg. % in the negative to as much as 20 to 30 mg. % in the positive.

Another factor which influences the time of onset of ketosis is the fluid intake. Ehrström [1922] draws attention to the influence of water hunger on the excretion of the ketonic acids. He cites the case of a middle-aged man who had vomited everything for 3 days and then developed a strong acetonuria and acetone-charged breath. Both these symptoms diminished in intensity when, without any food being given, an infusion of saline was carried out. He also refers to a similar experience with a case of pyloric stenosis. The polyuria of diabetes is also regarded by him as a contributory factor in the ketogenesis. He refers also to the acetonuria of dipsophobes.

We have not as yet tested this point fully but up to the present we can say that starvation ketosis appears more rapidly in warm weather and much more rapidly if no fluid is taken. It is, however, not to be supposed that there is any serious shortage in water in so short a period as two days. The body undoubtedly has considerable stores of water upon which it can draw before serious symptoms are produced. It is of interest to recall that the metabolism of 100 g. fat produces about 105 g. water whilst 100 g. protein give rise to about 40 g. water.

In the experiments to be described a considerable number of fastings were carried out and in most of them no fluid was taken for 36 hours. Such a fast can bring about a good deal of disturbance and in one case (Dr A. L.) the ketosis was so intense that some water had to be given, after which the condition was much improved. In general, however, an average man can starve for 36 hours even without fluid, with very little upset. Certain symptoms, however, frequently appear, as tendency to headaches, fatigue, difficulty in concentration and cramping pains in the muscles of the extremities. On one occasion an intense constricting pain was felt in the chest and this was followed by a sudden vertigo and fainting attack. It was found that the blood sugar at the time of these symptoms was down to 0·056 %. The cramping pains are probably of the same nature as those felt by cases of severe diarrhoea, with frequent large loss of fluid—the extreme cases being found in cholera. In spite of these symptoms, it was decided in our experiments to undergo the starvation without fluid in order to develop as considerable a ketosis as possible in as short a time as possible.

The ketosis of starvation differs in certain ways from that occurring in diabetics. Whereas in starvation the ketosis increases, in diabetics fasting brings about a marked diminution in the excretion of the ketonic substances. At first sight this would suggest a difference in mechanism in the two cases, a supposition which Joslin [1917] supports.

It seems, however, more reasonable to believe that the diminution in ketosis following starvation in the diabetic is due simply to the withdrawal of his high fat diet and in the normal to the removal of the relatively high carbohydrate diet. This view is taken by Labbé *et al.* [1921], Shaffer [1921] and Woodyatt [1916].

Although starvation will reduce ketosis in the diabetic it will not remove it entirely.

In both diabetic and starvation ketosis there is a deficiency in carbohydrate metabolism; in the former due to failure of the insulin mechanism, in the latter due to withdrawal of carbohydrate.

The analogy between the condition in starvation and that in diabetes can be extended further to include diminished tolerance to carbohydrate, low respiratory quotient, as well as ketosis. These facts will appear in the data presented below.

EXPERIMENTAL.

1. The plan of experiment was to undergo a preliminary period of complete starvation and then, having determined the fasting levels of R.Q., blood sugar and excretion of ketonic substances, to ingest solutions of the various sugars and make similar observations.

Having regard to the different constitution of the sugars and to the different degrees of storage and oxidation which have been observed after their ingestion, it was considered probable that they would show *in vivo* different antiketogenic powers.

Owing to the different solubilities of the sugars the degree of absorption from the gut is different for each sugar. Ordinarily sugar does not appear in the faeces to any considerable extent but it is certain that decomposition takes place in the intestine as a result of the activity of the intestinal flora.

2. To detect small quantities of acetoacetic acid we used the nitroprusside reaction.

3. To estimate the ketonic substances we used a method described by the writer elsewhere [1925].

4. Blood sugars were determined by MacLean's method.

5. For the determination of respiratory quotients Haldane's apparatus with the Douglas bag was used. Although a certain amount of movement is necessary in order to collect blood and urine, it is possible with some practice to carry this out without interfering appreciably with the R.Q.

6. The substances investigated were: glucose, fructose, mannose, sucrose, lactose, maltose, galactose and glycerol.

7. Except where otherwise stated the subject of experiment was the writer.

Exp. 1. In this experiment the sugar examined was pure *glucose*. A fast was commenced at 7 p.m. 30. xii. 24. The urine from 8 p.m. 31. xii. 24 till 9. a.m. 1. i. 25 was collected.

At 10.30 a.m. 1. i. 25 the fasting blood sugar and respiratory quotient were determined. At 11.20 a.m. 50 g. pure glucose in 150 cc. water were taken.

The findings are given in Table I.

Table I.

Period of starvation, 40½ hrs.

Time a.m.	Urine cc.	Nitroprusside test	Acetoacetic acid + acetone mg. (acetone)			β-Hydroxybutyric acid mg.			Blood sugar %	R.Q.	Fehling's test
			per cc.	Total	per hr.	per cc.	Total	per hr.			
9.0	215	+	0·134	28·8	2·2	0·88	189·2	14·6	.	.	–
10.30	23	+	0·105	2·4	1·6	0·93	21·4	14·3	0·113	0·73	–
10.45	3	+	.	.	.	.	.	.	.	.	.
11.15	14·5	+	0·197	2·9	5·7	0·567	8·2	16·4	.	.	–
11.20	50 g. glucose taken										
11.30	11	+	0·153	1·7	6·7	0·542	5·96	23·9	.	.	.
11.45	8	+	0·192	1·5	6·1	0·876	6·81	27·2	0·104	0·74	–
12.0	10·5	+	.	.	.	.	.	.	0·184	.	.
p.m.											
12.15	8·5	+	0·163	1·4	5·6	0·365	3·1	12·4	0·189	0·75	+
12.30	6·5	–	0·039	0·25	1·01	0·869	5·7	22·8	0·187	.	+
12.45	8·0	–	.	.	.	.	.	.	0·189	.	+
1.5	8·0	–	0·041	0·33	1·18	0·806	6·45	19·34	0·141	0·81	–
1.30	14·0	–	.	.	.	.	.	.	.	.	–
2.55	25	–	.	.	.	.	.	.	0·099	.	–
3.45	10	+	0·192	1·92	2·3	1·08	10·8	12·9	.	.	–

From this table it will be seen that

1. The nitroprusside reaction became negative 1 hour after the ingestion of the glucose and remained negative for a further 3¼ hours. The excretion of acetoacetic acid and acetone fell from about 6 mg. per hour to negligible quantities and then began to rise with the re-establishment of the nitroprusside reaction. The figures given for acetoacetic acid during the period of negative nitroprusside reaction can only be regarded as approximations, as the method of estimation was not sensitive for quantities below 0·1 mg. The actual content in acetoacetic acid during this period was probably lower than the figures given.

2. The excretion of β-hydroxybutyric acid throughout this experiment showed no tendency to fall, even during the period of negative nitroprusside reactions.

3. The blood sugar curve was definitely abnormal and the glycosuria which occurred never normally occurs in the writer after 50 g. glucose.

For comparison we give the figures obtained after 50 g. glucose under ordinary post-absorptive conditions.

Normal blood sugar curve following 50 g. glucose taken 3 hours after morning meal.

Time	Blood sugar %	Fehling's test on urine
11.41 a.m.	0·122	–
11.50 50 g. glucose taken		
11.55	0·129	–
12.15 p.m.	0·159	–
12.35	0·150	–
12.55	0·125	–
1.15	0·117	–

4. The fasting respiratory quotient was 0·73 and after the glucose it rose slowly to 0·81 in 1½ hours. Normally the rise following 50 g. glucose is

to 0·9 in 1 hour. It will further be observed that the rise in R.Q. occurred after the maximum blood sugar had been passed.

5. The period of negative nitroprusside reaction corresponded to that of the fall from the maximum blood sugar to the fasting level.

Exps. 2 *and* 3. These were carried out on two normal young men (Dr A. L. and Dr A. C.) who kindly volunteered to undergo the rather trying experiment. As these experiments were exactly similar to Exp. 1 it will only be necessary to summarise the findings.

Table II.

T_1 = Time to overcome existing ketosis.
T_2 = Time during which the keto-antiketogenic balance was preserved.

Exp.	Subject and period of starvation	Glucose g.	T_1 hours	T_2 hours	Blood sugar curve	Max. R.Q.
1	M.W.G. 40½ hrs.	50	1	3¼	Abnormal	0·81
2	A.L. 38	75	2½	1¾	,,	0·86
3	A.C. 40	50	1	3	,,	0·82

By abnormal blood sugar curve is meant a curve indicating a diminished tolerance to the particular sugar administered.

The followed facts were elicited from these experiments:

1. Glucose is very efficient as an antiketogenic agent, but the rapidity of control of a starvation ketosis varies in different subjects. As a result of several starvations and ingestion of 50 g. glucose after 40 hours the values of T_1 and T_2 were found to be remarkably constant at 1 and 3 hours, respectively, in the case of the writer. It was therefore considered that a basis for qualitative comparison of the antiketogenic values of the sugars was provided by such experiments in the same subjects.

2. The tolerance to glucose after starvation is markedly diminished. The blood sugar curve after starvation always shows an exaggerated rise and much delayed return to normal levels. Glycosuria commonly occurred after quantities of glucose well below the normal tolerance.

3. In each case the maximum R.Q. was such as to indicate deficient oxidation, and this value was attained in each experiment some time after the maximum blood sugar was reached.

4. The period of active antiketosis (negative nitroprusside test in urine) always corresponded to the period of fall in blood sugar.

The abnormalities in blood sugar curves following starvation and diets low in carbohydrate have been recorded by several observers. Kageura [1922] found that the hyperglycaemia in man following the ingestion of the Japanese test meal (100 g. rice + 2 eggs) was greater after a carbohydrate-poor diet than after a diet rich in carbohydrate. In some cases 100 g. rice produced glycosuria after a meat-fat period. He found that protein and fat were equally effective in lowering carbohydrate tolerance.

Greenwald [1924], however, working with dogs, found that a diet of protein does not lower carbohydrate tolerance to the same extent that a fat diet does.

This is comprehensible when we consider that 56 % of protein is potential carbohydrate whilst only 10 % of fat is capable theoretically of yielding sugar, though actually we have never detected a perceptible rise in blood sugar after administration of glycerol to a normal subject.

Southwood [1923] also found that there was marked diminution in carbohydrate tolerance after starvation and low carbohydrate diet. Injection of a small dose of insulin produced a readjustment of the blood sugar curve to almost normal.

Brailsford Robertson, commenting on Southwood's paper, takes the view that during starvation, and hence low blood sugar, there is diminished activity of the islet tissue in the pancreas and hence physiological rest to this tissue. This he considers to be the rationale of Allen's fasting treatment of diabetes.

This indeed seems very probable, particularly as the R.Q. following 50 g. glucose and even 75 g. glucose in none of our cases rose to 0·9 or over. This would certainly indicate diminished power of oxidation which evidently we must refer back to a diminished secretion of insulin.

Having regard to the modern conception proposed by MacLean and de Wesselow [1920] that a rise in blood sugar stimulates the glycogenic mechanism, it seemed probable that a period of starvation would bring about a diminution in storage power. This conception of hyperactivity of the glycogenic function was based on the fact that after a dose of carbohydrate the blood sugar curve in its fall often reached values below the fasting level. But it must be remembered that this does not always occur in normal conditions. After starvation we have not, in general, observed a fall below the fasting level.

The next sugar investigated was *fructose*. Two experiments were made exactly similar to those with glucose. In the second of these 50 g. glucose were ingested after the re-establishment of the nitroprusside test in the urine, in order to see if the absorption of the fructose had readjusted the disturbed carbohydrate metabolism. We tabulate the results as before.

Table III.

Exp.	Period of starvation	Fructose g.	T_1 hours	T_2 hours	Blood sugar curve	Max. R.Q.
1	39 hrs.	50	$1\frac{2}{3}$	1	Rose to 0·133	0·77
2	39	50	$1\frac{1}{2}$	1	,, 0·120	0·77
		followed by 50 g. glucose	1	3	Normal	—

The average normal fructose curve for the writer is given for comparison, the sugar having been ingested at the time of the first blood sugar determination.

Time (mins.)	0	30	60	90	120
Blood sugar %	0·110	0·130	0·120	0·105	0·101

We must therefore consider that the fructose curve after starvation is not appreciably changed from the normal. If we accept the view that the fructose curve is an index of the carbohydrate-storing power of the organism, then it would seem that after starvation storage power is normal. Unfortunately the fructose test is not unequivocal, but it would seem that deficient storage is not the principal cause of the lowered carbohydrate tolerance after starvation.

The results with fructose may be summarised as follows:

1. Fructose acts very efficiently as an antiketogenic agent, having overcome the ketosis in about $1\frac{1}{2}$ hours and preserved a balance for 1 hour.

2. The period of negative nitroprusside reaction in the urine corresponded with the period of fall in blood sugar, showing that active antiketogenesis takes place during storage and oxidation of the carbohydrate.

3. The blood sugar curve did not indicate any deficiency in storage power, and normal carbohydrate metabolism was re-established by the fructose as shown by a normal glucose curve after the fructose curve had returned to normal.

4. The respiratory quotients indicated deficient oxidation and the maximum R.Q. was reached some time after the maximum blood sugar was reached. Bornstein and Holm [1922] have also shown that there is a latent period between the administration of sugar and the rise in R.Q., which contrasts with the immediate rise in blood sugar. It would seem that a certain height of blood sugar must be reached before the mechanism of oxidation is brought into full play. When the requisite level is reached the insulin secretion is stimulated and oxidation proceeds rapidly.

The next sugar investigated was *galactose*. A preliminary starvation of 42 hours was undergone, at the end of which a considerable ketosis had appeared. As in the former experiments 50 g. of the sugar in 200 cc. water were taken.

The findings in this experiment may be summarised as follows:

1. The ketosis was not affected by the ingestion of galactose. The nitroprusside reaction remained positive and the quantitative results agreed with the qualitative findings, except for a tendency to a diminution in the hourly excretion; but complete keto-antiketogenic balance never occurred.

That the galactose was absorbed appears in the fact that a powerful galactosuria and a rise in blood sugar occurred during the experiment.

It would appear that this sugar is incapable, at least during the period of this experiment, of controlling the ketosis of starvation.

2. The blood sugar curve commenced at 0·08 %, rose in 1 hour to 0·135 % and regained the normal in a further 80 minutes.

3. The R.Q. rose from a fasting level of 0·704 to 0·736 an hour after the galactose was taken and fell again to 0·704 in another hour.

4. The urine showed a powerful reduction of Fehling's solution when the blood sugar was between 0·104 % and 0·135 %, and continued to do so for nearly 4 hours, long after the blood sugar had returned to normal.

5. 50 g. glucose administered after the galactose blood sugar curve had returned to normal, produced an entirely normal curve, showing that the galactose had readjusted the disturbed carbohydrate metabolism.

6. There is a diminution in storage and oxidation of this sugar after starvation.

Normally 50 g. galactose taken by the writer 3 hours after a meal produces practically no rise in blood sugar, but galactosuria occurs in less than 30 minutes after taking the sugar.

As a result of many experiments on normal subjects with 50 g. galactose we have concluded that it gives rise to a blood sugar curve comparable to that of fructose, has no definite threshold and filters through the kidney with ease.

A similar experiment was carried out with *lactose* after a preliminary starvation of 40 hours. The results were very similar to those obtained with galactose. The ketosis was unaffected by 50 g. lactose and even the administration of a further 50 g. 3 hours after the first dose failed to overcome the starvation ketosis.

There was some doubt whether the lactose was completely absorbed as a considerable diarrhoea occurred later, and 15 estimations of blood sugar at intervals of about 15 minutes showed no appreciable changes. Lactosuria, however, occurred 30 minutes after the first dose of lactose and continued throughout the experiment; some of the sugar, therefore, was certainly absorbed.

The question as to the normal tolerance to lactose is unsettled. Folin and Berglund [1922] consider that it has no threshold, since they obtained lactosuria after 30 g. lactose without any attendant rise in blood sugar. Others, however, have found no lactosuria after more than 100 g. In our experiments we have in general found lactosuria after 50 g. and it has been present at low levels of blood sugar, *e.g.* 0·102 %, 0·096 %. We must, therefore, subscribe to the view that lactose has no real threshold and can appear in the urine at any level of blood sugar.

The next sugar examined was *sucrose*. The preliminary period of starvation was 44 hours, after which 50 g. sucrose in 200 cc. of water were taken. The ketosis developed was of the same order as those in former experiments and the conditions were as far as possible identical.

The results may be summarised as follows:

1. The sucrose rapidly and effectively controlled the ketosis. In 1 hr. 2 mins. the nitroprusside reaction became negative and remained so for a period of 1 hr. 7 mins. This sugar, therefore, whilst overcoming the ketosis as rapidly as glucose or fructose, was unable to preserve the keto-antiketogenic balance for more than 1 hr. 7 mins.

The normal curve for the writer is as follows:

Time	Blood sugar %	
Fasting	0·100	
50 g. sucrose taken		
30 mins. later	0·145	No glycosuria
60 ,,	0·164	
90 ,,	0·153	
120 ,,	0·090	

2. The blood sugar curve showed the abnormal rise and extended period of fall which has been observed with glucose. The fasting level was 0·09 % and 44 minutes after the sucrose was taken the blood sugar was 0·206 % and took 2 hrs. 40 mins. to return to 0·111 %.

3. The R.Q. showed a rise from 0·71 to 0·83 62 minutes after the sugar was taken and 18 minutes after the blood sugar had reached a maximum. It subsequently fell to 0·71.

4. The period of negative nitroprusside reaction corresponded to the period of fall in blood sugar.

5. Glycosuria occurred at a blood sugar level of 0·206 % and continued until the blood sugar was 0·11 %.

A similar experiment was carried out using *maltose*, the initial period of starvation being 37½ hours.

The findings may be summarised as follows:

1. 50 g. maltose rapidly and effectively controlled the ketosis. The nitroprusside reaction became negative in 1 hr. 8 mins. and remained negative for a further 2 hours.

2. The blood sugar starting at 0·097 % rose to a maximum of 0·179 % in 1 hr. 13 mins. and reached the fasting level in a further 90 mins.: 50 g. maltose normally raise the blood sugar to 0·16 % in 30 minutes and it reaches the normal again in a further 60 minutes.

The deficiency in carbohydrate tolerance after starvation is thus again manifest, although maltose does not exhibit it as well as glucose or sucrose. The urine throughout the experiment showed no tendency to reduce Fehling's solution.

3. The nitroprusside reaction remained negative throughout the period of fall in blood sugar.

The next sugar investigated was *mannose* which differs from glucose only in the arrangements of the H and OH groups at the second carbon atom. In taste it is peculiar. At first it seems quite sweet but in a moment or two a quinine-like flavour appears.

In the experiment the preliminary period of starvation was 39 hours. The mannose produced a violent intestinal disturbance with diarrhoea. The ketosis was entirely unaffected and the blood sugar showed no significant change. No reduction of copper occurred in the urine. It would appear that the mannose was not well absorbed so that we cannot at present be certain of its efficacy as an antiketogenic agent.

The last experiment deals with the effect of *glycerol* on the ketosis of starvation.

It is generally stated that glycerol is capable of forming carbohydrate in the body and that it increases the excretion of sugar when administered to diabetics; it has also been claimed that glycerol exerts some antiketogenic action. In man 20 g. glycerol can be completely oxidised. On taking 27 g. in one dose, 0·5 to 1 g. appears in the urine. It seemed, therefore, probable that glycerol would be effective in overcoming starvation ketosis.

To test this a fast of 38 hours was undergone after which 100 g. glycerol in 200 cc. water were taken. After taking this solution a violent headache was experienced which made it almost impossible to continue.

Summary of findings:

1. A very pronounced diuresis was observed, the volume passed every 15 minutes being about 40 cc. as compared with an average of 9 cc. in former experiments.
2. The nitroprusside test in the urine decreased somewhat in intensity but the total excretion of ketonic substances did not diminish.
3. The blood sugar increased from 0·09 to 0·113 % in the first half-hour after the glycerol was taken and returned to 0·09 % an hour later.
4. There was a marked excretion of glycerol in the urine.

We conclude from this experiment that glycerol has no antiketogenic action and, in doses of 100 g., produces no marked change in blood sugar.

SUMMARY.

1. The antiketogenic activity of certain sugars has been examined by administration during starvation ketosis.
2. Glucose, fructose, sucrose, and maltose have an antiketogenic action, whilst galactose, mannose, lactose and glycerol are inert.
3. The deficiency in carbohydrate tolerance after starvation, has been examined. Evidence of some deficiency in storage power and oxidation after starvation has been presented. Normal conditions are re-established after the administration of carbohydrate.
4. The rise in respiratory quotient following the ingestion of a sugar has been shown to occur some time after the maximum blood sugar is reached.
5. The maximum antiketogenic action of the sugar, as evidenced by a negative nitroprusside reaction, has been shown to occur during the period of fall in blood sugar, *i.e.* during storage and oxidation of the sugar.

I wish to thank Professor MacLean, of St Thomas's Hospital, for much advice and encouragement during the prosecution of this work.

My thanks are due to Dr Alexander Lyall for much help in the estimations.

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CXL. BIOCHEMICAL AND HISTOLOGICAL STUDIES ON LIGNIFICATION[1].

PART I. THE NATURE OF LIGNIN: ITS PHYSIOLOGICAL SIGNIFICANCE AND ITS ESTIMATION IN TIMBERS.

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Introductory and General.

Among the secondary chemical changes taking place in the cells of higher plants, lignification has been commonly held to proceed at the expense of cellulose, giving rise to membranes composed of compound cellulose such as lignocellulose. We have no precise knowledge of how lignocellulose is formed, from what substance or substances it originates, and what is its physiological significance in plant economy. Still less do we know concerning its exact chemical nature and its relation to other plant constituents.

It may be resolved by suitable chemical treatments in such a way that the non-cellulose constituent, viz. lignin, is rendered soluble at the least expense, leaving behind a maximum amount of cellulose. Lignocellulose plays an important part in the soil. Thus the processes of decay to which it is subjected vary according to the aerobic or anaerobic conditions to which it is exposed. It is only the cellulose component however that disappears, being broken down into gaseous and other products, whilst the aromatic lignin component remains behind and accumulates in the soil, furnishing that valuable organic constituent—humus—which is largely responsible for the texture of the soil.

Much evidence, *e.g.* Bray and Andrews [1924] and du Toit [1924], exists in favour of the view that the humic matter of the soil is derived from lignin and not from carbohydrates. Closely related to the humic acids of the soil, we find in coal a series of ill-defined substances known as "ulmic acids."

The chemistry of wood substance, designated as wood lignin or wood fibre, and the various microchemical colour reactions, have been the subject of numerous investigations which are well summarised in Czapek's "*Biochemie der Pflanzen.*"

It has generally been considered that the different colour reactions obtained with wood are due to its aromatic constituents, whether free or combined. These colour reactions may be due to coniferyl alcohol or a substance or substances having a similar propenyl or hydroxypropenyl grouping; or they may be due to a substance of the nature of vanillin. There seems no doubt that the active grouping concerned in the coloration is of the nature of an aldehyde, and not of a quinone. This conclusion is supported by the work of Selivanoff [1891] and Nickel [1887, 1889] who first showed that the lignin reaction disappears on treating the wood with sodium bisulphite, or hydroxylamine.

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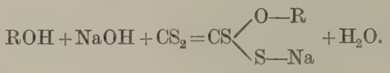
[1] Part of a Thesis approved for the Degree of D.Sc., University of London.

Czapek [1899] obtained a crystalline aldehydic substance which he termed "hadromal," giving all the colour reactions of wood, by treating wood with boiling stannous chloride solution. Hadromal is actually one of the aromatic constituents concerned partly in the lignification process and it is linked chemically with cellulose and other polysaccharides. The yield of hadromal does not exceed 1–2 % of the wood substance by the method employed by Czapek [1899]. On the other hand, the aromatic lignin component according to the methods hitherto proposed for its estimation is obtained in a yield of 10–30 % of the wood substance. The difficulty encountered in settling the hadromal question is due to the fact that so far we are not in possession of any method by which we can isolate this aromatic constituent exactly as it exists in combination with polysaccharides without destroying its reactive grouping. The methods employed for isolating the ligneous aromatic constituent in the above yield (10–30 %) depend on the treatment of lignified cell walls with 72 % sulphuric acid, concentrated hydrochloric acid, or caustic alkali which bring about certain changes. When we take all the properties of lignocellulose into account it appears to the writer that the aromatic constituent just before lignification is not of the nature of hadromal, but that it is produced by a secondary transformation of this aromatic component by processes of oxidation. At any rate the amount of the substance of the nature of hadromal thus produced is very variable even in the fully lignified cell wall.

Phloroglucinol is thus not a stain for the major part of the aromatic constituent which is the precursor of the one present in lignocellulose but rather for an aldehyde of the nature of hadromal. This aldehyde is always associated with lignin into which the major part of the aromatic substance formed first in the plant and not stained by phloroglucinol is converted. A test has been devised by the writer to which aromatic substances utilised in lignification respond readily. It is found that a 1 % solution of vanillin in concentrated sulphuric acid is a very sensitive reagent giving a bright-red coloration with such aromatic substances as are utilised in lignification. Further, the presence of these substances can be detected in parts not shown by the phloroglucinol reaction, and the localisation of the aromatic constituent by this reagent has thrown much light on the mechanism of lignification, for we can actually follow the migration and production of the aromatic substance in certain zones.

On the basis of many observations it was held by Erdmann and Baltzer [1873] and Cross and Bevan [1883–1895, 1] that cellulose is in ester-like combinations in wood. Wislicenus [1909, 1910], on the other hand, held that cellulose is only an adsorption compound with other substances contained in wood. Koenig [1912] also opposed the ester hypothesis since on treating wood with 72 % sulphuric acid and diluting with water he obtained a residue of lignin retaining the cell wall structure, whilst the cellulose was saccharified. Various reasons will be given in the present work in favour of the writer's view that lignin is present in combination with cellulose and certain polysaccharides as a glucoside. Our knowledge of the chemistry of lignocellulose we owe primarily to the valuable researches of Cross and Bevan [1895, 2–1916], where its properties are well summarised.

Lignocellulose does not take part directly in the cellulose xanthate reaction. Cellulose combines with sodium hydroxide, and the alkali cellulose on exposure to carbon disulphide reacts according to the following equation in which one of the hydroxyl groups of the cellulose molecule appears to react with greater facility than the others.



$$\mathrm{ROH + NaOH + CS_2 = CS}\begin{cases}\mathrm{O{-}R}\\\mathrm{S{-}Na}\end{cases}\mathrm{+ H_2O.}$$

The resulting compound, alkali cellulose xanthate, is perfectly soluble in water giving a very viscous solution. When lignocellulose from jute is submitted to this reaction a partial conversion (20–50 %) of the constituents of the fibre is observed. In this reaction it is the group designated by β-cellulose that takes a part and is in fact dissolved, whilst the undissolved residue possesses all the properties of the original lignocellulose. On the other hand the lignocellulose of wood is completely resistant to the cellulose xanthate reaction. The writer believes that this behaviour points to the fact that in the molecule of lignocellulose from wood both the α- and β-celluloses are in firm chemical union with the lignin component, whilst β-cellulose in those lignocelluloses which participate partially in the xanthate reaction is more probably a mixture of unlignified β-cellulose together with the true lignocellulose. Further, it is well known that of the three free hydroxyl groups in the molecule of cellulose there is one which is much more reactive than the others and is the first to take part in the thiocarbonate reaction. This leads the writer further to conclude that when this reactive hydroxyl group is already occupied or blocked through its linkage with the lignin component there exists a molecule of true lignocellulose composed of celluloses α- and β- linked chemically with the lignin constituent, which, as a whole, although containing free hydroxyl groups, is inert as far as the thiocarbonate reaction is concerned. This behaviour is not surprising as steric hindrance plays an important part in certain reactions in which the hydroxyl groups of polysaccharides are involved. If lignin were merely adsorbed on cellulose as held by Wislicenus and Kleinstuck [1910] we should expect it to be readily dissolved by the alkali of mercerising strength (17–18 %) used in the thiocarbonate reaction, as it is a powerful solvent for free lignin, and in addition, if the reactive hydroxyl group of the cellulose constituent were free, it should form the cellulose xanthate. As lignocellulose from wood does not take any part directly in the above xanthate reaction, the writer considers she is justified in concluding that the usual reactive hydroxyl group of the normal cellulose is linked chemically with the lignin component in lignocellulose.

Resolution of Lignocellulose.

Amongst the different methods employed for resolving lignocellulose, the chlorination process and the treatment with nitric acid have been employed, but these are too costly for industrial operation. The industrial processes of resolution are chiefly based on treatment with sulphites or bisulphites or alkalis. The recent work of Klason [1917, 1922, 1923], throws much light on the nature of the lignin constituent which is present as a soluble sulphonic derivative in the digested mixture. The sulphite lye after digestion has been shown to contain two ligninsulphonic acids corresponding to lignin-α and lignin-β.

The resolution with alkalis is effected with 7–10 % sodium hydroxide at a pressure of 6–10 atmospheres. The alkaline mode of resolution has many points to commend itself for critical scientific investigation, the results of which are described herein. It is very rapid and complete, and, from the point of view of investigating the lignin component, it has the advantage that this can be isolated in the full yield, without any radical decomposition of the aromatic nucleus, by simply acidifying the alkaline liquid, as it is insoluble in acid solutions or in water. From the crude precipitate, lignin can be isolated quantitatively by suitable purification. This has now been accomplished in a very simple manner, and conditions have been found under which the resolution of lignified plant materials can be accomplished readily, and further, the same conditions have been extended to the preparation and quantitative estimation of lignin.

When lignocellulose is boiled with a 5–10 % solution of caustic soda at the ordinary pressure the resolution does not proceed beyond 15–20 % of the total lignocellulose. On treating with alkali at higher pressures in an autoclave the amount hydrolysed rapidly increases with the pressure. When the pressure is kept constant and the strength of alkali is varied, hydrolysis increases with the concentration up to a certain limit, beyond which decomposition of lignin sets in. The preliminary experiments to establish these conditions were carried out with a homogeneous sample of sawdust from white deal as follows.

Five grams of the wood shavings or sawdust are accurately weighed out and transferred to a 200 cc. "durosil" test tube (2″ × 8″), containing 100 cc. of caustic soda solution of varying strengths and heated at pressures of 2·5 and 3 atmospheres for 1 hour. At the end of this period the contents are allowed to cool, made up to 250 cc. with distilled water and after thorough mixing, filtered through a filter paper. 50 cc. of the dark brown alkaline filtrate is carefully measured out with a pipette into a 100 cc. cylinder and acidified with 5 cc. of concentrated hydrochloric acid. The precipitated lignin, which is accompanied by traces of other substances, is allowed to settle and filtered through a small Buchner funnel. It is well washed with cold distilled water until the filtrate as tested with silver nitrate is free from chlorides. The lignin is readily freed from the accompanying impurities by dissolving it in boiling 95 % alcohol in which the impurities are insoluble. The precipitate together with the filter paper is transferred carefully to a beaker so that it rests flatly on the bottom. It is treated repeatedly with boiling 95 % alcohol just sufficient to cover the paper and the alcoholic solution carefully filtered into a weighed flask. The extraction is repeated until the alcoholic solution is quite colourless, and any lignin remaining on the filter paper is thoroughly washed out with boiling alcohol. The alcoholic filtrate is evaporated to dryness and weighed to constant weight as lignic acid. On the extraction of the precipitated crude lignin with alcohol a trace of insoluble matter, chiefly composed of cellulose-β giving all its reactions, remains behind [Powell and Whittaker, 1924]. Results of experiments with sawdust from white deal with varying concentrations of alkali at 2·5 and 3·0 atmospheres are given in the following tables.

Concentration of sodium hydroxide	% lignic acid obtained at		% lignocellulose resolved at	
%	2·5 atmos.	3 atmos.	2·5 atmos.	3 atmos.
1·0	5·40	5·90	28·52	31·22
2·5	5·90	7·00	31·22	37·04
4·0	6·10	7·30	32·27	38·62
5·0	5·80	6·90	30·68	36·51
7·5	4·90	5·60	25·93	29·63
10·0	4·20	5·10	22·22	26·98
15·0	3·40	4·40	17·99	23·28

The above results show that the maximum amount of resolution was produced with 4 % sodium hydroxide solution. In the next series therefore concentration was kept constant, and the pressures varied up to 16 atmospheres using 4 % NaOH solution. The results are given in the following table.

Pressure in atmospheres	% lignic acid obtained	% lignocellulose resolved
Normal pressure	2·50	13·23
1	4·60	24·34
2	5·05	26·72
3	7·40	39·15
4	8·67	45·87
5	10·50	55·55
6	12·70	67·19
7	14·30	75·66
8	15·50	82·01
9	17·70	93·65
10	18·90	100·00
12	19·20	—
16	19·20	—

The foregoing results show that the resolution of the lignocellulose is practically complete under the conditions mentioned at 10 atmospheres. As this pressure corresponds with a temperature of 180°, it was considered advisable not to exceed it as ordinary resistant cellulose remains practically unattacked at that temperature. In view of these results a few experiments were carried out to ascertain if it were possible to reduce the pressure below 10 atmospheres and obtain complete resolution by slightly extending the period of digestion from 1 hour to 1·5 hours. The results obtained were as follows.

The percentage of lignic acid obtained at 8 atmospheres with 4 % NaOH solution after 1 hour and 1·5 hours' digestion was 15·6 and 17·1 respectively. Similarly the percentage of lignic acid obtained at 10 atmospheres after 1 hour and 1·5 hours was 18·9 and 18·95 % respectively. These preliminary experiments were further sufficient to show that the resolution was complete at 10 atmospheres in 1 hour whilst at 8 atmospheres even after 1·5 hours the percentage resolved did not approach that at 10 atmospheres and 1 hour.

Estimation of Lignin.

Having arrived at these conditions of complete resolution it was thought worth while to investigate if the principle outlined above could not be applied to the elaboration of a reliable method for estimating lignin in plant materials, as the present methods for the determination of lignin are not reliable or suitable for carrying out a large number of determinations. In the first place, it was necessary to ascertain if the lignic acid suffered any decomposition when treated with alkali at 10 atmospheres. For this purpose, varying amounts of lignic acid were dissolved in 20 cc. of 4 % sodium hydroxide and digested at 10 atmospheres for 1 hour. The results were as follows:

Weight of lignic acid taken. g.	Weight of lignic acid obtained after digestion. g.
0·055	0·052
0·110	0·108
0·165	0·162
0·220	0·221

The results show that under the above conditions lignic acid does not suffer appreciable decomposition and the procedure was extended therefore to the determination of lignin in different woods. It will be here unnecessary to review the methods which have hitherto been proposed for the estimation of lignin. An examination of the proposed methods [Konig and Rump, 1914; Konig and Becker, 1918; Willstätter and Zechmeister, 1913; Krull, 1916; Benedikt and Bamberger, 1890; Herzog, 1896; Cross, Bevan and Briggs, 1907; Seider and Hempel, 1907] is sufficient to show that the difficult problem of the estimation of lignin has not yet been solved in a satisfactory manner.

Having now shown that lignocellulose can be resolved quantitatively by heating it with 4 % sodium hydroxide for 1 hour at 10 atmospheres, and that the lignin can be recovered quantitatively after such treatment without any primary decomposition, a direct gravimetric method has been devised on the basis of these results for estimating lignin.

The procedure is as follows. 5 g. of the wood is accurately weighed out in a thick glass durosil test tube 8″ × 2″ and heated with 100 cc. of 4 % sodium hydroxide solution at 10 atmospheres for 1 hour. The solution after cooling is made up to 250 cc. and filtered. The lignic acid is then estimated in an aliquot volume of the filtrate (50 cc.) according to the process already described. From the amount of lignic acid the percentage of lignin can be readily calculated. The procedure has been applied to the determination of lignin in a large number of woods and has been found to give most consistent results.

When we come to examine the nature of lignin we shall see that, like all other secretory products of constructive metabolism, it is present in varying amounts in different woods partly in the free condition directly extractable by alcohol, the remaining major portion in chemical combination with the cellulose hydrolysable by alkali.

Accordingly, the estimations of lignin have been carried out on unextracted and extracted timbers, giving the total and combined lignin respectively, whilst the difference corresponds with the free lignin. The results of these estimations are given in the following table, and in a few cases (sufficient for the purpose) are also compared with those obtained by the method of Konig and Becker [1918].

It will be seen that the results obtained by the latter method are decidedly higher than those given by the alkali method. Since the estimation of lignin by treatment with concentrated hydrochloric acid (45 %) is well known to give results concordant with those obtained by 72 % sulphuric acid, only the latter method was employed for comparison. The values hitherto recorded for the percentage of lignin obtained by the last two methods are too high for the following reasons. In the first place, it must be admitted that lignin obtained by these two methods is not pure. It is also assumed that no secondary reactions occur and that the carbohydrates are completely removed. Hagglund [1923, Hagglund and Bjorkman, 1924] in a series of papers giving the results of a critical examination of the nature of lignin obtained by the concentrated hydrochloric acid method has shown that the lignin always retains a portion of hexosan and pentosan residues amounting to 10–12 %, which is constant over a very prolonged period of treatment.

The writer also observed on critical examination of the lignin prepared by treatment with 72 % sulphuric acid that it is not only associated with carbohydrate residues amounting to 4·0–6·0 %, but that it suffers sulphonation during the treatment. This is proved by the fact that after carefully washing the lignin free from sulphates it gives, on ignition with pure sodium carbonate, sulphate amounting to 12·0–14·0 % SO_4. The values obtained by these methods can therefore hardly be considered as reliable.

Estimations of total, combined and free lignin in timbers.

Timbers	Total lignin 4 % NaOH %	Total lignin 72 % H_2SO_4 %	Combined lignin 4 % NaOH %	Free lignin 4 % NaOH %
Lignum Vitae	38·39	47·99	15·57	22·82
Teak	31·07	45·80	22·66	8·41
Rose Wood	27·28	41·10	11·89	15·39
Red Deal	26·40	35·70	20·41	5·99
Mahogany	24·22	33·95	19·66	4·56
Pitch Pine	23·77	33·80	17·90	5·87
Oregon Pine	21·11	36·70	18·73	2·38
Larch	21·33	32·80	18·33	3·00
Oak	21·10	31·20	17·33	3·77
Yellow Pine	20·30	32·08	18·70	1·60
White Deal	20·50	29·10	18·70	1·80
Elm	19·32	27·00	17·32	2·00
Ebony	18·97	52·10	15·73	3·24
Box Wood	18·50	26·29	14·44	4·06
Spruce	17·80	30·55	16·98	0·82
Black Wood	17·06	—	7·72	9·34
Parana Eucalyptus	16·30	—	13·03	3·27
Sandal Wood	16·11	—	14·55	1·56
Walnut	16·01	—	11·29	4·72
Canary Wood	15·27	—	11·60	3·67
Plane Wood	14·36	—	9·90	4·46
English Oak	14·14	—	13·17	0·97
Ash	11·93	—	11·05	0·88
Birch	10·48	—	9·66	0·82
Beech	9·32	—	9·00	0·32

Micro-estimation of lignin. There is as yet no reliable micro-method for the estimation of lignin and, being specially interested in the study of its distribution, the writer next directed her attention to elaborating a colorimetric method for its estimation. There was no difficulty in such micro-work as far as the resolution of lignocellulose was concerned and the problem was to estimate the lignin present in the alkaline extract obtained under the conditions described above for its macro-estimation. It was first decided to make use of the marked phenolic properties of lignin in such micro-estimation by coupling it in alkaline solution with an excess of a diazotised aromatic amine. The amount of the dye produced is then under proper conditions proportional to the amount of lignin.

The amine is dissolved in 10 parts of water containing the equivalent quantity of hydrochloric acid. The solution is cooled to 0° and a saturated solution of the calculated quantity of sodium nitrite is added all at once. The solution may be tested after 5 minutes with potassium iodide paper to ensure an excess of nitrite. To carry out the coupling, an alkaline solution of the phenolic substance (*e.g.* lignin) after cooling to 5–10° is treated with an excess of the diazotised base. Care is taken that the phenol solution should contain sufficient alkali and that the mixture does not become acid. On the addition of the diazotised amine, a dye is produced which can be employed for colorimetric estimation. A few preliminary estimations were conducted on the above lines by coupling lignin in alkaline solution with diazotised aniline, *p*-nitraniline, *m*-phenylenediamine, *α*-naphthylamine and benzidine.

Lignin gave rise to dyes having a reddish brown tinge with the first three diazotised bases, whilst α-naphthylamine and benzidine gave deep red colours. Of these two, benzidine was selected as the more suitable as the colour was much more permanent than that obtained with α-naphthylamine. The diazotised benzidine solution was prepared as follows. One g. of benzidine was dissolved in 10 cc. of water containing 2 cc. of concentrated hydrochloric acid and diazotised with a saturated solution containing 0·8 g. sodium nitrite.

Benzidine was particularly suitable compared with other amines because, with these, traces of the corresponding phenols are produced during diazotisation according to the following equation if the temperature is not sufficiently low:

$$R . N_2 . Cl + H_2O = R (OH) + N_2 + HCl.$$

The phenol thus produced itself gives a colour on adding alkali. With benzidine however, this was not observed. A standard solution of lignin was prepared by weighing out 0·1 g. of pure anhydrous lignin, dissolving in a little alkali and making up the solution to 250 cc. To carry out the colorimetric estimation 1 cc. of the above lignin solution = 0·4 mg. lignin was accurately measured out with a 1 cc. pipette, graduated to 0·01 cc., in a 100 cc. Nessler cylinder containing 2 cc. of 4 % sodium hydroxide. To the mixture was added 0·5 cc. of the diazotised 1 % benzidine solution and after diluting to about 50 cc. it was left at the ordinary temperature for half an hour. The volume was then made up to 100 cc. and the tint compared with that obtained in a solution containing an unknown amount of lignin. In order to test the accuracy of the above method, experiments were first conducted with known amounts of lignin, varying from 0·1–1·0 mg. The results of these experiments showed that they were always reliable so long as the tint of the standard or the unknown solution was within 95 % of the other solution. This necessitated the preparation of a large number of standards, and attempts were therefore made in another direction to improve the estimation on the same principle. A solution of phosphotungstic and phosphomolybdic acids in phosphoric acid is the most sensitive reagent we possess for estimating minute amounts of aromatic substances containing hydroxyl groups, which in a solution made alkaline with sodium carbonate give a deep pure blue coloration with the above reagent. The reagent was first introduced by Folin and Denis [1912] for the colorimetric estimation of tyrosine. It is prepared by dissolving 100 g. sodium tungstate, 20 g. phosphomolybdic acid and 50 cc. of 85 % phosphoric acid in 750 cc. of water. The solution is boiled under a reflux condenser for 2 hours, cooled and made up to 1000 cc. A standard solution of lignin in dilute alkali was prepared by weighing out 0·5 g. of pure anhydrous lignin and dissolving it in about 25 cc. of 4 % sodium hydroxide solution; it was then carefully made up to 500 cc. so that 1 cc. = 1 mg. lignin. A few qualitative tests showed that the reagent gave a beautiful blue colour with quantities of lignin varying between 1·0–0·01 mg. The standard conditions employed for the colorimetric estimation are as follows. Known volumes

(1·0–0·1 cc.) of the standard lignin solution are accurately measured out into Nessler cylinders and to each is added 2·5 cc. of the phosphotungstic phosphomolybdic reagent and after 5 minutes 12·5 cc. of a saturated solution of sodium carbonate. The volume is made up to 100 cc. and the colours matched with that obtained under similar conditions from an unknown amount of lignin. Experiments first undertaken with known quantities of lignin showed the method to be thoroughly reliable so long as the depth of the colours under comparison did not differ by more than 20 %. The results were also in close agreement with those obtained by the gravimetric method.

DISTRIBUTION OF LIGNIN IN WOOD.

The method was first employed in studying the distribution of lignin in the autumn and spring wood of Oregon pine and spruce. The autumn and spring wood were first separated by cutting a thin shaving of uniform thickness of the wood along the borders of the bands of the autumn and spring tracheides. The relative weights of the autumn and spring tracheides separated in the above manner from Oregon pine were found to be approximately 2 : 1 whilst from spruce they were in the ratio of 1 : 2 respectively. 0·1–0·2 g. of the cuttings containing the autumn or the spring wood was digested with 20 cc. of 4 % NaOH at 10 atmospheres for 1 hour. The digested liquid was then made up to 100 cc. with distilled water and filtered. As the alkaline extracts thus obtained contained substances other than lignin, also giving a coloration with the phosphotungstic-phosphomolybdic reagent, it was necessary to separate the lignin from these.

A known volume (1·0–5·0 cc.) of the digested liquid is accurately measured out into a 15 cc. centrifuge tube and acidified with a few drops of concentrated hydrochloric acid. The precipitated lignin is centrifuged, washed twice with distilled water and dissolved in a few drops of 1 % sodium hydroxide. The alkaline solution is carefully washed into a Nessler cylinder and the colorimetric estimation carried out as already described.

In order to determine whether there was any decomposition of lignin under the conditions employed for the digestion in the micro-estimation, quantities of lignin varying between 5–30 mg. were dissolved in 20 cc. of 4 % sodium hydroxide and heated in an autoclave for 1 hour at 10 atmospheres pressure. The solutions were then made up to 100 cc. and the lignin estimated colorimetrically in an aliquot portion as described, but no diminution in the quantity of lignin was observed.

Distribution of Lignin in Autumn and Spring Wood of Spruce and Oregon Pine. (Dry.)

	Lignin %	
	Autumn wood	Spring wood
Spruce	17·7	15·1
Oregon pine	14·4	18·6

The above results are the average of a number of estimations.

The same method was next applied to the study of the distribution of lignin in different parts of Scotch fir (*Pinus sylvestris*). The results are given in the following table. The twigs were further subdivided into four concentric rings so as to study the lignin content in separate layers.

Percentage of Lignin in different parts of Scotch Fir.

	Lignin % on green	Lignin % on dry	Moisture %
Leaf	5·10	8·63	40·9
Buds	1·90	9·4	79·7
Thick twig 14 years old: cortex	1·79	5·44	56·5
outer ring	11·11	14·29	24·9
inner ring	15·50	23·08	34·9
centre	8·57	11·69	26·7
Thin twig 4 years old	8·34	15·90	47·6
Thin twig 2 years old: end portion	6·17	9·56	35·5
bottom portion	7·82	12·12	35·5

Percentage of Lignin in different layers of stems of Hawthorn, Ash and Diseased Ash.

	Lignin %			
	Cortex	Outer ring	Inner ring	Centre
Hawthorn	14·07	8·94	11·37	10·24
Diseased ash	12·53	13·71	—	15·30
Normal ash		Average 11·93		
Diseased ash		,, 13·80		

The above results show that there is no marked variation in the lignin content of the autumn and spring wood. The lignin content of the buds is striking in that they contain practically the same amount of lignin as the older parts. The lignin content of the green buds is only 1·9 % owing to the high moisture content. This indicates that lignification commences at a very early stage simultaneously with the thickening of the cell wall.

The woods are known in nature to be susceptible to two types of attack by organisms known as white rot and brown rot. It is believed that in the former decomposition of lignin is induced leaving the cellulose behind, whilst the latter type of rot proceeds at the expense of cellulose leaving lignin undecomposed.

The greater percentage of lignin in different layers of a stem of slightly diseased ash (brown rot) compared with the average percentage in normal ash suggests that a preferential decomposition of cellulose probably takes place.

THE NATURE AND PROPERTIES OF LIGNIN.

Lignin as prepared under the conditions described for its estimation is obtained as a light brown powder, insoluble in water but soluble in dilute alkalis, ammonia, alcohol, acetone and acetic acid. It reduces ammoniacal solution of silver nitrate slightly and Fehling's solution still less. When boiled with hydrochloric acid it does not give rise to furfural, hence it is quite free from polysaccharide units, and its characteristics agree with the generally accepted view that it is wholly an aromatic compound. It has a pleasant aromatic odour distinctly recalling vanillin in some cases. When an alcoholic solution of it is evaporated to dryness in a flat dish, it is obtained in the form of a highly lustrous brown film. It melts at 167–169° to a clear brown liquid without decomposition and with a highly purified product the M.P. did not rise above 170°.

Lignin possesses faintly acidic properties which are more pronounced than those of the polyhydroxy phenols. It dissolves in cold dilute sodium carbonate solution from which it is reprecipitated on acidification. Its acid value—*i.e.* the number of grams of lignin dissolved by a litre of N NaOH is 477. Lignin also possesses many of the characteristics of an unsaturated compound. Its iodine value as determined by Wijs' method is 139.

The determination of the acid value of lignin presented unusual difficulties, as, owing to its feeble acidity and the dark colour of its solution, it was impossible to carry out a titration using indicators in the ordinary way. The procedure adopted was as follows: 0·1432 g. of finely powdered anhydrous lignin was accurately weighed out in a small beaker and suspended in about 10 cc. of distilled water. The suspension was heated on a water-bath and 29·1 cc. of $N/100$ sodium hydroxide were gradually run in until a small portion of the lignin remained undissolved. The undissolved lignin was then filtered off, washed thoroughly with distilled water and estimated gravimetrically. The difference between this amount and the quantity of lignin taken, 0·1432–0·0040 = 0·1392 g., corresponds with the lignin dissolved by 29·1 cc. of $N/100$ NaOH. Hence the acid value is 477.

The iodine value which is an indication of the degree of unsaturation of lignin was determined as follows. 0·1 g. of pure anhydrous lignin was dissolved in a little glacial acetic acid in a well stoppered flask together with 25 cc. of Wijs' solution. The stopper was then moistened with potassium iodide solution and the mixture kept in the dark for 45–60 mins. A blank experiment with Wijs' solution was also performed simultaneously under the same conditions. 20 cc. of 10 % potassium iodide solution and 100 cc. of water were then added and the excess of iodine titrated with $N/10$ sodium thiosulphate using starch solution as indicator in the usual way.

Titre of the blank experiment = 49·3, 49·3 cc. of $N/10$ $Na_2S_2O_3$
Titre of the actual ,, = 38·2, 38·4 ,, ,,
Average amount of iodine absorbed by 0·1 g. of lignin; (49·3–38·3) × 0·0127 = 0·1397 g. Iodine value = 139·7.

Lignin prepared by the method described above behaves as a homogeneous compound, because on purification in different ways its properties remain unchanged.

It gives very characteristic almost insoluble normal and basic calcium and barium salts, prepared by adding a solution of barium or calcium chloride to an ammoniacal solution of lignin. The content of calcium or barium of such salts varies according to the strength of the ammonia solution used for dissolving the lignin. When precipitated from a strongly ammoniacal solution the percentage of calcium or barium is very much higher. The procedure generally followed for the isolation of these salts was as follows. The ammonia solution used for dissolving a known weight of lignin was first carefully freed from carbon dioxide to avoid the subsequent contamination of calcium or barium lignate with the carbonates. To the boiling ammoniacal solution of lignin, calcium or barium chloride was added in excess, when the lignin was precipitated as an amorphous dirty brown precipitate. It was washed free from salts and dried. The salt is absolutely insoluble in water, soluble in acetic acid, dilute sodium hydroxide solution, or alcoholic hydrochloric acid, and the lignin can be recovered unchanged in its properties from such salts. One remarkable fact which will be noticed from the following results is that 95–98 % of the lignin is precipitated as the insoluble alkaline earth salt. This would be very unlikely if the lignin were not of a homogeneous nature. The results of the analysis of calcium, barium and sodium salts are given below. The ammoniacal salt cannot be prepared in the solid form as it dissociates on concentration and the lignin is precipitated as such.

	Salt	Conc. of ammonia %	g. of lignin taken	g. of salt obtained	g. of sulphate obtained by ignition with H_2SO_4	% of metal in salt	Yield % of theoretical
1	Ca	5	0·4052	0·4215	0·1325	Ca, 9·20	95
2	Ca	1	0·2830	0·2950	0·0840	Ca, 8·40	97
3	Ba	5	0·1400	0·2050	0·1375	Ba, 39·4	104
4	Na*	—	0·1392	0·1498	0·0213	Na, 4·6	102

* Prepared by treating an excess of lignin with a minute quantity of NaOH under conditions described for the determination of acid value.

The alkaline earth salts do not appear to have a definite composition owing to their tendency to form basic salts. An alcoholic solution of lignin when treated with a dilute solution of ferric chloride does not give any coloration but yields a greyish brown precipitate.

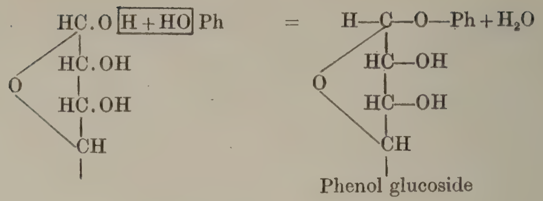
Having found that lignin is capable of forming insoluble compounds with the metals of the alkaline earths, it was of interest to examine some woods in order to ascertain whether any of the lignin was present in this form. This was determined by first removing the free lignin by extraction with alcohol, and, the calcium lignate being easily soluble in cold alcoholic hydrochloric acid, the loss in weight after treatment with the latter corresponded with the amount of lignin present as insoluble lignate. The exact procedure employed is given below.

An accurately weighed amount of wood (2·0–2·5 g.) in the form of thin shavings was extracted with 90 % alcohol in a Soxhlet extractor for 4½ hours and the loss in weight was determined after drying the extracted wood to constant weight. The extracted wood in a glass thimble was dried and then placed overnight at the ordinary temperature in a 1 % solution of hydrochloric acid in 90 % alcohol. It was then well washed free from acid with 95 % alcohol, again submitted to extraction with 90 % alcohol in a Soxhlet extractor for $4\frac{1}{2}$ hours and the loss in weight corresponding with the amount of insoluble lignate determined by drying the extracted shavings to constant weight. The results showed that lignin is not present in woods to any appreciable extent as insoluble lignate.

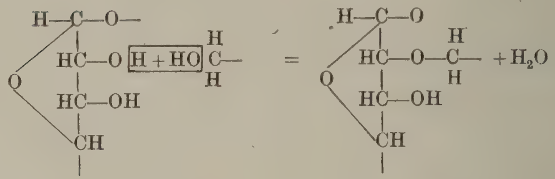
CONSTITUTION OF LIGNOCELLULOSE.

Having shown that lignocellulose is a compound in which lignin is chemically combined with cellulose, and is not an adsorption mixture of cellulose incrusted with lignin, the experiments next to be described were undertaken with a view of determining the nature of the linkage between the two constituents in lignocellulose. When we take into consideration the nature of the reactive groupings in cellulose and lignin the possibilities narrow down to two only. In the first place we have to remember that celluloses isolated from lignocelluloses, unlike the unlignified celluloses of the cotton type, always show reducing power to some extent. The latter therefore contain only carbinol groups, whilst in the former we have to recognise in addition the presence of reactive carbonyl groups. In the lignin component, on the other hand, we have highly reactive phenolic hydroxyl groups which are partly acetylated and also methylated. Next in importance is the carboxyl group in lignin which as we have seen represents a very small portion of the molecule. In addition, attached to the benzene nucleus, is an aliphatic chain containing carbinol, aldehydic and unsaturated groups, but as these are not of primary importance in determining the nature of the linkage, they will not be considered here. Essentially, therefore, we have to find out the types of possible linkages between the reactive groupings in the two components of lignocellulose. In the first place, the reactive carbonyl group in the cellulose component of lignocellulose can react with the phenolic hydroxyl group of the lignin component giving rise to a type of compound widely disseminated in nature, viz. phenolic glucosides, saponins and glucotannoids. On the other hand, if this reactive carbonyl grouping were absent, as it is from cotton cellulose, glucosidic linkage would not be possible. The hydroxyl groups in polysaccharides, which are relatively not so reactive as the carbonyl groups, can react with aliphatic and aromatic substances containing the primary alcohol group on the one hand, and the carboxyl group on the other. Substances formed by the linkage of the hydroxyl groups of the polysaccharides with the primary alcohol group of aliphatic and aromatic substances are not commonly found at all in nature. This is only what might be expected as the affinity between these two groups is by no means strong. The carboxyl groups of aliphatic and aromatic substances have on the contrary a considerable affinity for hydroxyl and carbonyl groups of polysaccharides and give rise to compounds which are frequently found in nature, *e.g.* tannins. These considerations may be graphically summarised as follows:

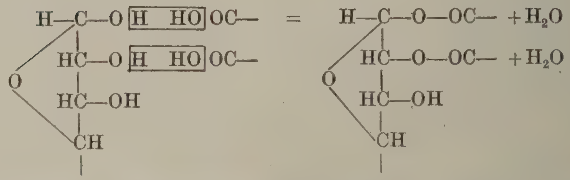
I.



II.



III.



It is now necessary to decide between linkages I and III. The earlier workers who were against the adsorption hypothesis of lignocellulose, Erdmann and Baltzer [1873] and Cross and Bevan [1883–1895] suggested that the linkage in lignocellulose is of an ester type as in III. There are however strong reasons against the ester type of linkage in lignocellulose. In the first place a linkage of this type is only possible when one of the constituents possesses marked acidic properties. The writer has already shown that lignin does not possess strongly acidic properties. Further, lignocellulose, when submitted to hydrolytic treatment under conditions by which other esters of the polysaccharides are known to be hydrolysed readily, does not behave in the same manner. Such esters when heated with very dilute mineral acids or even water under pressure are split up readily into their components. Boiling with glacial acetic acid and treating with oxidising agents such as potassium permanganate, potassium dichromate and hydrogen peroxide are also very effective hydrolytic agents.

Lignocellulose from which free lignin was removed by extraction with alcohol was heated with 20 times its weight of water and 0·5 % sulphuric acid at 10 and 6 atmospheres respectively for 1 hour. The extract was then thoroughly washed out and the residue heated with 4 % sodium hydroxide in a boiling water-bath to dissolve the lignin produced on hydrolysis. The residue from the alkaline extract was washed thoroughly and dried. The unhydrolysed lignin was then estimated in the manner already described. The results were as follows.

The residual lignocellulose after treatment with water at 10 atmospheres for 1 hour contained 49·36 % of the total lignin, the remainder, 50·64 % having been hydrolysed into its components.

After treatment with 0·5 % sulphuric acid at 6 atmospheres for 1 hour, 67·5 % of the total lignin was hydrolysed.

On boiling lignocellulose with five times its weight of glacial acetic acid for three hours only 22·73 % was hydrolysed.

When lignocellulose was treated with small quantities of potassium permanganate there was practically no hydrolysis although with larger quantities a diminution in the lignin content was observed due to its oxidising action.

These treatments are sufficiently drastic and are known to hydrolyse readily and completely the linkage between hydroxyl and carboxyl groups in the different derivatives of polysaccharides. The glucosidic linkage is on the other hand much more resistant. All the evidence available at present points to the presence of a glucosidic linkage in lignocellulose. This is further substantiated by the fact that aromatic substances which take part in lignification migrate from one part to another in the plant as glucosides. The writer has so far searched unsuccessfully for enzymes which would be likely to bring about the hydrolysis of lignocellulose. It is possible that lignin is in combination with the reactive modification of dextrose, the glucosides of which are not split up by the usual glucosidolytic enzymes.

Cellulose as a Component of Lignocellulose.

It has already been mentioned that when lignocellulose is hydrolysed with 4 % sodium hydroxide under the conditions described for the estimation of lignin, a residue of resistant cellulose is obtained of a high degree of purity. Unlike the residue obtained from other methods of resolution of lignocellulose it possesses an exceptionally light colour, being even snow-white in some cases. Chemists and physiologists have for a long time been in doubt as to what is to be understood by pure cellulose, and until we arrive at some definite conception of the purity of cellulose, the problem of its estimation will remain empirical. The writer does not consider that any of the numerous methods which have been proposed for the estimation of cellulose are accurate as will be seen from the considerations set forth below.

Isolation of Cellulose-α. Cellulose has been held by Cross and other workers to exist in raw cellulosic materials in three forms: cellulose-α, -β, and -γ. The distinction is more or less empirical and is based on the relative resistance to the specific solvent action of strong caustic soda. Cellulose-α is not attacked by 17·5 % sodium hydroxide solution, whilst cellulose-β and -γ both pass into solution. Cellulose-β is further differentiated from cellulose-γ in that it is precipitated on acidifying the alkaline solution with acetic acid. The separation is by no means sharp and is determined very largely by the physical condition and penetrability of the material.

The approximate estimation is carried out according to the method described by Cross and Bevan [1916]. To compare the behaviour of different cellulosic materials when treated with 4 % sodium hydroxide at 10 atmospheres for 1 hour with that of 17·5 % sodium hydroxide at the ordinary temperature as employed by Cross and Bevan, a few preliminary experiments were carried out as a result of which a sharper separation of cellulose-*α*, uncontaminated with other celluloses has now been effected. Three materials were selected for these experiments, viz. cotton wool, raw untreated cotton fibre and sulphite paper pulp. These were treated with 17·5 % sodium hydroxide. Cotton wool according to this method gave only 2 % of cellulose-*β* and -*γ* whilst raw untreated cotton was not found to contain either cellulose-*β* or -*γ*. These materials (1·0 g.) were next heated with 4 % sodium hydroxide (25 cc.) at 10 atmospheres for 1 hour. The results are given in the following table.

	Treatment with 17·5 % NaOH		Treatment with 4 % NaOH at 10 atmospheres	
	% cellulose-α	% cellulose-β and -γ	% cellulose-α	% cellulose-β and -γ
Cotton wool	98·01	1·99	83·00	17·00
Raw cotton	100·00	0·00	82·52	17·48
Sulphite pulp	68·50	No cellulose-β	57·20	42·80

The cellulose-*α* obtained after treating the cotton wool with 4 % sodium hydroxide was treated repeatedly with this reagent for 1 hour periods at 10 atmospheres to ascertain its resistance, the loss in weight being determined after each treatment.

Loss suffered by cellulose-α obtained by treating cotton wool with 4 % *sodium hydroxide at* 10 *atmospheres pressure for* 1 *hour.*

	Loss in weight %	Resistance value %
2nd treatment	5·54	94·46
3rd ,,	6·96	93·04
4th ,,	5·31	94·69
5th ,,	5·62	94·38
Average	5·86	94·14

Experiments were next carried out to ascertain whether the percentage weight lost in the above treatments was affected by varying the relative volume of 4 % sodium hydroxide solution, but no variation was observed in several experiments. These results, taken generally, show that cellulose-*α* suffers an average loss of 5·86 % on heating at 10 atmospheres for 1 hour with 4 % sodium hydroxide; and, after making due correction for this, the true percentage of cellulose-*α* in the material can easily be deduced. The results further show that treating the cellulosic material with 17·5 % sodium hydroxide does not effect a sharp separation.

The corrected percentages of cellulose-*α* are given in the following table.

	% cellulose-α corrected (dry)
Cotton wool	88·17
Raw cotton	87·66
Sulphite pulp	60·76

ESTIMATION OF CELLULOSE-α IN PLANT TISSUES.

Cellulose-α prepared by treatment with 4 % sodium hydroxide under the conditions described is the purest type of cellulose yet isolated.

This conclusion is substantiated by the results of a critical microscopical examination of the stain-taking capacity of celluloses-α, -β and -γ prepared by the method of Cross and Bevan and cellulose-α prepared by the writer's method. It has been found that cellulose-β and -γ are stained by certain dyes which do not stain cellulose-α and yet stain the cellulose-α obtained by the method of Cross and Bevan. This shows that cellulose-α prepared under the conditions employed by Cross and Bevan is associated with cellulose-β and -γ which are not completely removed. Cellulose-α has therefore been estimated by the writer's method.

Estimation of Cellulose-α in Timbers.

	% cellulose-α corrected (dry)
White deal	44·71
Spruce	43·34
Red deal	40·80
Plane wood	34·95
Rose wood	34·50
Black wood	29·79
Box wood	28·73

Distribution of Cellulose-α and Lignin in Elm (Ulma campestris).

	Cellulose-α (corrected)		Lignin		
Parts	Moist wood %	Dry wood %	Moist wood %	Dry wood %	Moisture %
Cortex	26·40	30·54	21·28	24·66	13·68
Phloem	27·49	45·44	1·20	1·98	39·46
Sap wood	18·42	22·31	7·90	9·56	17·44
Heart wood	23·11	34·26	7·27	10·69	32·56

Estimation of Cellulose-α and Lignin in Hemp and Flax.

	Cellulose-α corrected (dry) %	Lignin (dry) %
Russian Siretz hemp	57·01	1·47
Italian hemp	50·03	0·26
Russian Rhine hemp	47·80	1·46
Polish hemp	45·47	2·80
Spanish hemp	63·52	0·36
Siberian hemp	56·72	2·50
Kentucky hemp	43·45	0·23
Russian Petchure flax	42·47	0·62
Dew-retted Belgian flax	53·33	0·95
Irish flax	52·05	1·73

Agricultural chemists have long been trying to find the factors which operate in the lodging of crops, and in this connection it was thought of interest to determine the cellulose and lignin content of straws of cereals grown under definite cultural conditions. The results are interesting and show that whenever the true cellulose content of the tissue is low, there is a marked tendency to lodging. Straw from Plot 2, receiving farmyard manure only, is interesting compared with straws from plots receiving artificial fertilisers in that, although it contains the highest percentage of lignin, it does not make up in stiffness for the lower content of cellulose. Barley straw No. 9 obtained from a water culture contains the lowest percentage of both cellulose and lignin.

The results of these estimations together with the history of the straws are given below. The straws were obtained through the kindness of Sir John Russell from the Rothamsted Experimental Station.

Estimations of Cellulose-α and Lignin in Straws grown under different cultural conditions.

		Cellulose-α (corrected) %	Lignin %
(1)	Plot 2 B	24·66	12·40
(2)	,, 3	24·68	8·43
(3)	,, 6	32·24	7·98
(4)	,, 7	27·15	7·31
(5)	,, 8	28·04	8·67
(6)	,, 12	27·71	8·77
(7)	,, 13	34·93	8·09
(8)	,, 16	35·37	8·57
(9)	,, 9	17·52	4·40
(10)	Indian maize	37·06	6·55
(11)	,, rice	37·99	4·88
(12)	,, savannah grass	36·01	5·88
(13)	,, wheat	34·41	7·81

HISTORY.

(1) Broadbalk wheat, plot 2 B, farmyard manure; almost flat in places.
(2) Broadbalk wheat, plot 3, no farmyard manure; individual stacks laid.
(3) Broadbalk wheat, plot 6, minerals plus single S/A (Sulphate of Ammonia); not laid.
(4) Broadbalk wheat, plot 7, minerals plus double S/A; somewhat laid.
(5) Broadbalk wheat, plot 8, minerals plus treble S/A; very badly laid.
(6) Broadbalk wheat, plot 12, S/A, super phosphate plus Na_2SO_4; more prone to laying than plot 7; sometimes opposite holds.
(7) Broadbalk wheat, plot 13, S/A, super phosphate plus K_2SO_4; less prone to laying than plot 6; sometimes opposite holds.
(8) Broadbalk wheat, plot 16, minerals plus double N/S (Nitrate of Sodium); becoming laid.
(9) Barley straw from water culture, Serial No. 43, grown with no added SiO_2.
(10) Indian maize.
(11) Indian rice.
(12) Indian savannah grass.
(13) Indian wheat.

SUMMARY.

Lignocellulose is neither an ester-like nor an adsorption compound, as shown by the xanthate reaction. From the considerations of the reactive groupings of the constituents of lignocellulose, lignin seems to occur in chemical combination with cellulose and related polysaccharides as an aromatic glucoside.

The aromatic constituent just before lignification is not of the nature of hadromal, as shown by its negative reaction with phloroglucinol and its positive reaction with vanillin in concentrated sulphuric acid. Vanillin is the most sensitive reagent for localising the aromatic substances which are later

converted by oxidation into substances of the nature of hadromal, which combine directly with cellulose to give lignocellulose and give positive reactions with phloroglucinol and negative with vanillin.

Lignocellulose is resolved completely into its constituents by heating with 4 % sodium hydroxide at 10 atmospheres for 1 hour, without any radical decomposition of the lignin constituent, which can be precipitated by acidifying the alkaline liquid and isolated in the pure condition quantitatively by extraction with alcohol.

Lignin occurs in wood in a condition partly extractable by alcohol, but the major part is in combination with polysaccharides which can be resolved by alkali.

A reagent consisting of phosphotungstic and phosphomolybdic acids in phosphoric acid is a most sensitive reagent for estimating colorimetrically minute quantities of lignin, with which it gives a deep blue coloration in presence of sodium carbonate solution. A study of the distribution of lignin in different parts of Scotch fir by the colorimetric method shows the presence of lignin in young twigs, as well as in leaves and buds.

Lignin as isolated from wood is a brown, amorphous, faintly acidic substance with a pleasant aromatic odour, recalling vanillin in some cases, and melting at 170°. It is insoluble in water, soluble in dilute alkalis, and alcohol. Its iodine value is 139, and acid value 477. It gives insoluble salts with alkaline earth metals which do not possess a definite composition owing to the tendency to form basic salts.

When lignocellulose is resolved by the sodium hydroxide method a residue of pure resistant cellulose-α is obtained which can be estimated accurately.

The writer in conclusion feels it a great pleasure to express her sincere gratitude to Prof. A. R. Ling and Dr D. R. Nanji for their valuable criticism and for their continuous encouragement and interest in the course of these investigations. For a supply of specimens of different timbers her thanks are also due to Mr J. Harris whose practical knowledge has been of great value.

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CXLI. BIOCHEMICAL AND HISTOLOGICAL STUDIES ON LIGNIFICATION[1].

PART II. HISTOLOGICAL STUDIES ON THE POLYSACCHARIDES AND AROMATIC CONSTITUENTS OF THE CELL WALL.

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INTRODUCTION.

The section of organic chemistry dealing with the carbohydrates is perhaps of more absorbing interest to the plant physiologist than any other, since it deals with the infinite variety of substances composing the tissues of plants. For a long time the botanist had regarded "cell wall" and cellulose as synonymous terms, but as our knowledge of these constituents developed, it became more and more apparent that the cell wall presented such a considerable differentiation of form and substance as to render the classification and relation of these constituents to one another extremely difficult. It is surprising to find that in spite of the enormous advances in our knowledge of the chemical constituents of the cell wall, plant histology has remained more or less empirical and it is not characterised by that degree of exactitude which should be attainable with the chemical knowledge now at our command.

The writer has therefore undertaken these investigations primarily with the object of developing the necessary technique and of furnishing such data as would be helpful in studying the chemical differentiation of the substances comprising cell walls, so as to systematise plant histology on more rational lines.

This has necessarily involved a considerable amount of fundamental work, which should prove of particular interest to those desiring to undertake critical histological work, since at present we do not know exactly the nature of a given constituent stained by a dye, or how the staining capacity of one constituent is affected by its association with another.

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[1] Part of Thesis approved for the degree of D.Sc., University of London.

ISOLATION AND PROPERTIES OF THE PRINCIPAL CONSTITUENTS OF THE CELL WALL.

It will be useful in following the subject under discussion to review briefly the methods adopted by the writer for the isolation and preparation of the principal constituents of the cell walls of plants and their essential characteristics as disclosed by our chemical knowledge.

Celluloses.

In the first group comprising typical cotton cellulose, which is a seed hair, we shall include all fibrous celluloses which constitute the bast of exogenous flowering annuals: *e.g.* the celluloses of flax (*Linum usit.*), hemp (*Cannabis sativa*), China grass (*Rhea* and *Boehmeria* species). Celluloses of this group, when freed from the other associated substances by suitable chemical processes known to be within the limits of resistance of the true cellulose, have not so far been differentiated. They are very resistant to hydrolysing and oxidising agents, and do not possess reactive carbonyl groups.

Closely related to the above group, we have another class of celluloses which are much more widely distributed in the vegetable kingdom. These are differentiated from the first group by containing a slightly higher percentage of oxygen, and by the presence in them of carbonyl and methoxy groups. They appear to be formed from the celluloses of the first group by partial depolymerisation and oxidation.

Among the different types of cellular constituents we have to recognise three distinct types of celluloses, cellulose-α, -β and -γ, possessing different degrees of dispersion and dehydration, and corresponding polymerisation. The essential difference between them, as already mentioned in Part I of these researches, is that the less resistant celluloses, -β and -γ, are soluble in 17·5 % sodium hydroxide solution at the ordinary temperature, and from the alkaline solution cellulose-β is precipitated on acidification with acetic acid, whilst the cellulose-γ remains in solution. The writer has found that this method of separation is not quite sharp for isolating absolutely pure cellulose-α, and has therefore adopted another method for its preparation.

Cellulose-α is prepared by treating cotton wool (5·0 g.) with 100 cc. of 4 % sodium hydroxide at 10 atmospheres pressure for one hour. By this means it is completely freed from cellulose-β and -γ. It is then washed with water and dilute acetic acid, finally washed again thoroughly with distilled water and dried.

Cellulose-β and -γ are prepared from cotton wool by the method of Cross and Bevan [1916].

Cellulose-γ is isolated from the acid filtrate, obtained after filtering off cellulose-β, by submitting it to dialysis to remove mineral matter. It is then concentrated under reduced pressure to a suitable volume and cellulose-γ precipitated by the addition of two volumes of 95 % alcohol. To study their staining capacity they are best kept suspended in alcohol; a thin film is made by drying a drop of the suspension on a slide.

These celluloses show considerable differences with different stains. (See Table I.)

Hydrocellulose and Oxycellulose. Closely related to the above three cellular constituents we shall next consider their degradation products, which are frequently found in normal cell walls. These degradation products may be divided into two classes: (1) those produced by partial hydrolysis and (2) those produced by oxidation. The first group is typified by hydrocellulose which possesses active carbonyl groups, and shows a very strong adsorptive power for different organic dyes. The writer finds that there are two kinds of hydrocellulose, which, although staining similarly with different dyes, can be differentiated with aqueous iodine in potassium iodide solution. One modification takes immediately a deep blue colour whilst the other is only stained a reddish brown. The latter can be prepared from cotton wool according to Girard's method [1881].

The second modification is prepared by soaking cotton wool with as small a quantity as possible of cold 72 % (by vol.) sulphuric acid and treating almost immediately with 95 % alcohol. The hydrocellulose is then washed free from acid with distilled water and dried in the air. It stains a deep blue with aqueous iodine in potassium iodide solution. (See Table I for staining reactions.)

Under the term "oxycelluloses" we find comprised in the literature a number of compounds of indefinite character which are produced by the action of different oxidising agents on cellulose. These should not be regarded as entities but merely as representing mixtures of oxidised cellulose, hydrocellulose and unchanged cellulose, the difference being only due to the different degrees of surface oxidation of the fibres.

Oxycelluloses possess acidic properties, and also contain active carbonyl groups. There are three types of oxycelluloses, known as α-, β- and γ-oxycellulose, which are differentiated by their behaviour towards dilute sodium hydroxide solution, ammonia and water. α-Oxycellulose is difficultly soluble in alkali and insoluble in ammonia. β-Oxycellulose is soluble in dilute alkali and ammonia, whilst γ-oxycellulose is soluble in hot water, alkali and ammonia solution. α-Oxycellulose was prepared according to the method of Murumow, Sack and Tollens [1901]; β- and γ-oxycelluloses were prepared according to the method of Nastjukoff [1900]. (See Table I for staining reactions.) Oxycellulose may be detected in presence of hydrocellulose and celluloses according to the method of Schwalbe and Becker [1921] and Knagg [1908].

Among the non-fibrous polysaccharide constituents of the cell wall, we shall now consider in turn, mannan, galactan, pectin, amylohemicellulose, hemicelluloses, gums, starch, lichenin and chitin. This class is characterised by the low resistance of its members towards hydrolytic agents; they are more or less soluble in dilute alkali. The members are also readily broken down in the plant under the action of its own enzymes, secreted under abnormal conditions. The cell walls of amylaceous seeds especially are composed in part of polysaccharides of this class.

Mannan. Mannans like celluloses also occur in nature in different degrees of polymerisation, but they give mannose instead of glucose on hydrolysis with mineral acids. A variety of mannan termed paramannan which corresponds in many respects with cellulose-α, in that it is very resistant to hydrolytic reagents, is found in coffee beans and *Phytelephas macrocarpa.* It is not acted on appreciably by mineral acids, potassium chlorate and hydrochloric acid, but dissolves in a concentrated hydrochloric acid solution of zinc chloride. Hence it is frequently called mannocellulose. It can be readily separated from the less resistant mannan by boiling it with dilute alkali, in which the latter dissolves. Vegetable ivory, the endosperm of the palm (*Phytelephas macrocarpa*) was selected as the most suitable material for the isolation of mannan. It was boiled in the form of fine shavings with 1 % sodium hydroxide solution and the residue, consisting of mannocellulose, was washed with dilute acetic acid and distilled water, and dried. The less resistant mannans are found in leaves, roots, and stems of many plants, as well as in the cell walls of many fungi.

Mannans form insoluble compounds with copper in alkaline solution and hence are not soluble in Schweitzer's reagent. When they are in association with cellulose they also prevent it from dissolving. The less resistant mannan is isolated according to the method of Salkowski [1894]. With different stains it behaves mostly like gums. (See Table I.)

Galactan. Galactans are strictly analogous to mannans, giving rise to galactose instead of mannose on hydrolysis. They also appear to exist in varying degrees of polymerisation as some of them are not dissolved by boiling with alkali. They also form insoluble copper compounds and behave in most of their properties like the mannans. Galactans are often found in combination with pentosans such as xylan or araban. Galacto-araban is widely disseminated in the cell walls of the cotyledons of many plants, *e.g. Coffea arabica*, *Cocos nucifera* and other palms. Agar-agar of commerce, which is the mucilage from certain genera of the red seaweeds (*Rhodophyceae*), is the most suitable material for the isolation of this constituent. (See Table I for staining reactions.)

Pectins. Pectic substances are closely related to galactan, being derived from it by oxidation processes. In fact they constitute, in more or less intimate association with cellulose, the middle lamella of cell-walls in many tissues, in some only at a certain period of their life history. They occur abundantly in many succulent fruits, and also in fleshy roots and stems.

The most recent investigations of Nanji, Paton and Ling [1925] have cleared up the uncertainty which hitherto existed as to the nature of these substances by devising methods whereby these compounds have been isolated in pure state, and their nature and relationships to one another determined. (See Table I for staining reactions.)

Amylohemicellulose. Amylohemicellulose is generally associated with starch in the starch-bearing seeds and roots. It gives a blue coloration with iodine in potassium iodide solution, is insoluble in water and is attacked by secretion, but not by translocation, diastase. It is isolated from cereal starches by a method based on this fact, as shown by Ling and Nanji [1925].

It is a white amorphous powder, swelling up in boiling water, and is readily soluble in dilute sodium hydroxide solution. (See Table I for staining reactions.)

Hemicellulose. The hemicellulose constituents of woods sometimes give a dirty brownish blue coloration with iodine in potassium iodide solution. They are sparingly soluble in water, easily in dilute sodium hydroxide solution and are readily hydrolysed by boiling with dilute mineral acids. They are prepared from wood according to the method of O'Dwyer [1923]. (See Table I for staining reactions.)

Gums. Gums are very varied in nature and occur widely distributed among plants, especially trees. They are to be regarded as being derived from the decomposition of pectic substances in the plant. They also consist of polysaccharide acids in combination with hexoses and pentoses, such as galactose, arabinose, and xylose. They are of an acidic character, and occur mostly in nature in the form of salts of potassium, calcium or magnesium. (See Table I for staining reactions.)

Starch. Starch, although not forming a constituent of the cell wall by itself, may often be confused with amylohemicellulose and the hemicelluloses of woods already described, because of the coloration observed with iodine solution in the living cells of the pith, medullary rays and cortex of roots and stems. (See Table I for staining reactions.)

Lichenin. Lichenin, or moss starch, forms the principal constituent of Iceland moss (*Cetraria Islandica*) and Irish Moss (*Chondrus crispus*). It gives a dirty blue coloration with iodine and is insoluble in cold water. It can be best isolated by boiling the commercial dried moss with water, when it swells up, giving a viscous paste which sets to a jelly on cooling. (See Table I for staining reactions.)

Chitin. Chitin has long been recognised as a constituent of the cell-wall of certain fungi, *e.g. Boletus edulis*, *Agaricus campestris*, etc. It is very resistant to alkali and this fact is made use of in isolating it from the other constituents. It is insoluble in Schweitzer's reagent, gives a reddish brown stain with iodine in potassium iodide solution, and with a 50 % solution of zinc chloride-iodine it gives a bluish violet coloration. It is best isolated from the shells of the crustacea, lobster or crab, according to the methods of Gilson [1894] or Winterstein [1894, 1895]. When finely powdered and extracted successively with boiling alcohol and ether it is obtained as a white powder. (See Table I for staining reactions.)

Lastly, we have to consider the aromatic constituents of the cell-wall.

The isolation and properties of lignin have already been dealt with in Part I of this work. It may now be mentioned that while the writer was in search of some specific microchemical tests for lignin, it was observed that there exists some close relationship between lignin and resin, as it gives all the reactions of the latter. When dissolved in a little acetic anhydride and mixed with sulphuric acid (sp. gr. 1·53), it gives a deep red colour, changing through green to brown like the resins [Morawski 1889]. When treated with a drop of 1 % solution of vanillin in concentrated sulphuric acid, it gives a deep orange-red colour—violet in some cases—changing after 2–4 hours to reddish brown or bluish violet, a behaviour similar to that of the resins. This view was further confirmed by the similarity in the behaviour of lignin and resin towards different stains. Chemically, resins are not well-defined substances. They are for the most part solid or semi-solid substances of a gummy nature, originating in special cells in the plant. Resinous substances have solubilities and elementary composition similar to those of lignin. There are no good specific reactions for the microscopical detection of resins and essential oils. Resins can therefore only be identified by their physical properties, such as solubility, colour and other characteristics. In the plant resins appear in the liquid form as strongly refractile, homogeneous or vascular-foaming drops, or in solid yellowish brown amorphous masses. Alkannin from alkanna roots, first employed by Muller [1866], stains resins or free lignin and essential oils an intense red colour. Lignin and resins are also deeply stained by malachite green, borax carmin, safranine and ruthenium red.

Procedure followed in the staining experiments.

The period of staining was 2 hours, at the close of which period the excess of the stain was washed off thoroughly first with 90 % alcohol and finally with absolute alcohol and the preparation mounted in cedar wood oil, the cover-glass being ringed round with Canada-balsam. Cedar wood oil was found to be the most suitable general mounting medium as the depth of the stain was not affected. A list of the stains used and their compositions are given below.

(1) Phloroglucinol—1·0 g. phloroglucinol dissolved in 100 cc. of 12 % hydrochloric acid (1·060).

(2) Malachite green (alcoholic)—1 g. in 300 cc. of 92 % alcohol.

(3) Cobalt thiocyanate—One volume of saturated cobalt nitrate solution added to two volumes of saturated ammonium thiocyanate solution.

(4) Congo red (aqueous)—1 g. in 200 cc.

(5) Borax carmin (alcoholic)—3·0 g. carmin, 12 g. borax, 480 cc. water and 480 cc. 90 % alcohol.

(6) Eosin (alcoholic)—1 g. in 300 cc.

(7) Safranine (alcoholic)—3 g. safranine 500 cc. water and 500 cc. 90 % alcohol.

(8) Haematoxylin (Delafield)—200 cc. of a saturated solution of ammonium alum, mixed with a solution of 2 g. haematoxylin in 12 cc. absolute alcohol.

The mixture is exposed to light and air for a week. It is filtered and to the filtrate are added 50 cc. of glycerol and 50 cc. of methyl alcohol. It is allowed to stand in sunlight until the full colour develops and is refiltered for use.

(9) Gossypimin (aqueous)—2 g. gossypimin, 900 cc. water and 60 cc. 90 % alcohol.

(10) Aniline blue picrate—3 g. aniline blue, 2 g. picric acid dissolved in a litre of water.

(11) Iodine green (aqueous)—1 % solution.

(12) Ferric thiocyanate (aqueous)—1·0 cc. of saturated ferric chloride to 10 cc. of 10 % ammonium thiocyanate solution.

(13) Ruthenium red (aqueous) 1 in 10,000.

(14) Alkannin (alcoholic)—alkanna roots are extracted with just sufficient amount of 90 % alcohol to cover the roots. The alcohol solution is decanted and filtered for use.

(15) Picro-ponceau (aqueous)—0·1 g. ponceau in 100 cc. of saturated aqueous picric acid.

(16) Orcein (alcoholic)—0·5 % solution in alcohol containing 0·01 % hydrochloric acid.

(17) Vanillin—1·0 g. in 100 cc. of concentrated sulphuric acid.

In the following table the intensity of a stain taken up by a particular constituent is invariably based on the deepest stain obtained with that dye as the standard. It is of course evident that the intensity of the stain would vary according to the density of the material, but so long as due allowance is made for this and the conditions kept as nearly constant as possible, fairly reliable judgment can be given after a little practice as to the intensity of the stain. (See Table I.)

All the results of staining given in Table I hold good for direct staining only. Many of the stains taken up by a constituent can be displaced by another stain having a stronger affinity for the particular constituent. The writer in the course of her work, while trying to differentiate the various constituents of plant tissues by counterstaining has accumulated some useful data, which are epitomised in Table II. Generally the section is first stained with the most permanent primary colour and secondly with the counterstain for a suitable period. Borax carmin and malachite green, aniline blue picrate and gossypimin, haematoxylin and safranine or gossypimin are usually employed.

In addition to these the writer has used a number of pairs of counterstains with sections of timbers with good results for critical differentiation. (See Table III.) Many of these counterstains are especially good for differentiating the middle lamella.

It will be evident from a superficial examination of Table I that most of the stains are not at all specific as they stain a number of different constituents, and this fact has led to many misconceptions. For example, ruthenium red which is generally regarded as a specific stain for pectic sub-

Table I. *Showing the depth of stain taken up by different constituents of the cell wall.*

	Phloroglucinol in HCl.	Alcoholic malachite green	Cobalt thiocyanate	Aqueous Congo red	Alcoholic borax carmin	Alcoholic eosin	Alcoholic safranine	Haematoxylin	Aqueous gossypimin	Aniline blue picrate	Aqueous iodine green	Ferric thiocyanate	Aqueous ruthenium red	Alcoholic alkannin	Aqueous picro-ponceau	Alcoholic orcein
Cotton wool	—	—	—	+4	+2	+4	+1	+1	+1	+3	+1	—	—	—	+2	+1
Untreated natural cotton wool	+2	+1	—	+3	—	+3	+3	+1	+3	+3	+1	—	+1	—	+2	—
Natural cotton wool treated with acid	+½	+2	—	+3	+1	+3	+3	+1	+1	+3	+1	—	+1	—	+2	+1
Natural cotton wool treated with alkali	—	+2	—	+4	+1	+2	+1	+1	+1	+3	+1	—	—	—	+2	+1
Cellulose-α	—	—	—	+4	—	—	+½	+1	+½	—	—	—	—	—	—	—
Cellulose-β	—	+½	—	+4	—	+1	+2	+2	+½	+2	+½	—	—	—	+4	—
Cellulose-γ	—	—	—	—	+1	+2	+2	+2	+4	+2	—	—	—	—	+1	—
α-Oxycellulose	—	—	—	+4	—	+4	+4	+1	+4	+2	+2	—	+2	—	+2	—
β-Oxycellulose	+½	+2	—	+3	—	—	+4	+1	+4	—	—	—	+3	—	+1	—
γ-Oxycellulose	—	—	—	+3	—	+1	+2	+1	+1	—	—	—	+3	—	—	—
Hydrocellulose	+1	+4	+2	+4	+2	+2	+4	+4	+4	+3	+3	—	—	—	+2	+3
Hemicellulose	—	—	—	+4	+3	+4	+3	+4	—	+1	—	—	+4	—	+2	—
Gum	+4	+4	—	+3	+3	+4	+4	+4	+4	+3	+4	+3	+4	—	+1	—
Pectinogen	+1	+3	—	+3	+3	+4	+3	+1	+3	+2	+4	—	+4	—	—	— +1
Calcium pectate	+1	+3	—	+4	+2	+3	+4	+1	+4	+2	+3	—	+4	—	—	+2
Ferric pectate	—	—	—	+3	+2	+3	+3	+2	+4	+3	+4	+1	+3	—	+4	—
Lignin	—	+3 +4	—	+½	+3	— +1	+4	—	+1 +2	—	+2	—	+2	+3	—	— +1
Calcium lignate	—	+3	—	+3	+3	+3	+4	+1 +2	+3	+4	+3	—	+3	—	+1 +2	—
Galactan	—	+4	—	+2	+1	+4	+4	+1	+4	+2	+2	—	+4	—	—	—
Starch	—	+4	+3	—	—	+1 +2	+4	— +1	+3	+2	+2 +4	+3	+1	—	+½ Yellow	+½ Yellow
Mannan	—	+4	—	+4	—	— +4	— +2	+1 +2	— +3	+4	—	—	—to+3	—	— +1	— +2
Chitin	—	—	—	+4	+1 +3	+3	+1 +3	+1 +2	+1	—	—	— +1	— +1	—	+3	— +2
Lichenin	—	+2	—	— +2	+3	+2	+4	+2	+4	+4	+4	—	+4	—	—	—
Amylohemicellulose	—	+3	—	+4	+1	+4	+4	+2	+3	+4	+1 +3	+1	+2	—	+3	+1
Cork	— +4	+3 +4	—	—	+2 +3	—	—	—	—	+2 +3	+1	+1	—	—	—	—

— Negative. +1, +2 +3, +4, show increasing intensity of colour, +4 being the strongest.

Table II. *Showing the parts stained by different counterstains.*

Root. Malachite green stains: xylem and developing lateral rootlets.
Borax carmin stains: cortical tissue, endodermis and pith.
Haematoxylin stains: ground tissue, phloem, cortical tissue, cambium, resin duct and lactiferous vessels.
Gossypimin stains: xylem, endodermis and starch granules.
Safranine stains: secondary and primary wood, endodermis, central axis of root and lateral developing rootlets.

Stem. Malachite green stains: phloem, xylem elements, traces to first leaf, sclerenchymatous ring, cambiform tissue, spiral vessels, primary vascular bundles, bundle sheath, annular vessels, scalariform vessels, sclerenchyma sheath, nodal diaphragm and secretion cells.
Borax carmin stains: Primary ground tissue, cambiform tissue, pith, cortical tissue, phloem, primary medullary ray, epidermal tissue, endodermis and nucleated cortical tissue.
Haematoxylin stains: cortical tissue, primary ground tissue, central axis and medullary ray.
Gossypimin stains: phloem, xylem, primary medullary ray, cambiform tissue, resin canal, developing branch, thickening band of prosenchyma, starch granules and vascular bundles.
Safranine stains: xylem elements, phloem, connective tissue and pitted vessels.
Aniline blue picrate stains: cortical tissue, cambiform tissue, primary ground tissue, central ground tissue and secreting cells of canal.

Leaf. Malachite green stains: epidermis, hypodermis, resin duct, xylem, leaf hair and vascular bundle.
Borax carmin stains: parenchyma, endodermis, pericycle (transfusion tissue), phloem, epidermis, phloem of bundle and primary ground tissue.
Gossypimin stains: epidermis, sclerenchymatous fibres, xylem elements, stomata, small vascular bundles, cambium, phloem elements and cuticle.
Aniline blue picrate stains: ground tissue, phloem elements of bundle, palisade layer containing chloroplasts, guard cells of stomata, primary ground tissue, oil gland, epidermis, assimilating tissue, mucilaginous lining of the cell, nucleus and cuticle.

Table III. *Results of different counterstains with sections of Pitch Pine (timber).*

Phloroglucinol counterstained by malachite green:
Parts stained by phloroglucinol: autumn wood.
,, malachite green: spring and summer wood, cell remains, resin canal, medullary ray.
Congo red counterstained by malachite green:
Parts stained by Congo red: resin canal.
,, malachite green: cell wall, cell remains, medullary ray.
Congo red counterstained by cobalt thiocyanate:
Parts stained by Congo red: cell remains, medullary ray, resin canal.
,, cobalt thiocyanate: cell wall.
Ferric thiocyanate counterstained by malachite green:
Parts stained by ferric thiocyanate: autumn wood, tracheidal medullary ray.
,, malachite green: spring and summer wood, cell remains, medullary ray, resin canal.
Ferric thiocyanate counterstained by aniline blue picrate:
Parts stained by ferric thiocyanate: tracheidal medullary ray, cell wall, middle lamella.
,, aniline blue picrate: cell remains, medullary ray and resin canal.
Phloroglucinol counterstained by aniline blue picrate:
Parts stained by phloroglucinol: cell wall.
,, aniline blue picrate: cell remains, medullary ray and resin canal.
Gossypimin counterstained by malachite green:
Parts stained by gossypimin: middle lamella.
,, malachite green: cell wall, cell remains, medullary ray, and resin canal.
Malachite green counterstained by ponceau:
Parts stained by malachite green: cell wall, resin ducts, medullary ray.
,, ponceau: middle lamella.
Ponceau counterstained by aniline blue picrate:
Parts stained by ponceau: cell wall, middle lamella.
,, aniline blue picrate: cell remains, medullary ray, resin canal.
Alkannin counterstained by aniline blue picrate:
Parts stained by alkannin: tracheidal medullary ray.
,, aniline blue picrate: cell remains, primary medullary ray, resin canal.
Alkannin counterstained by malachite green:
Parts stained by alkannin: tracheidal medullary ray.
,, malachite green: cell wall, cell remains, medullary ray, resin canal.

stances and their derivatives is found to stain in addition α-, β- and γ-oxy-celluloses, hemicelluloses, gums, galactans, free lignin, mannan and amylo-hemicellulose. Moreover, there are no specific microchemical colour reactions for these constituents so that there is no direct means of differentiating between them even in this manner. On the other hand, many of the constituents can be removed from tissues by specific solvents or suitable chemical treatment and this principle has been employed by the writer in the work now to be described on the microchemical structure of the leaf and stem of *Pinus sylvestris*. Supposing it is desired to localise the pectic substances in different parts of a tissue, and sections of it be treated with specific solvents such as 0·5 % oxalic acid or 0·5 % ammonium oxalate respectively, both the treated and untreated sections being stained with different stains, a negative result will be obtained with some stains in certain parts of the treated sections compared with the untreated ones. Wherever a certain part is not stained after a specific treatment to remove a constituent, whereas the same part is stained in the untreated section, it is possible to say with certainty that those parts which do not stain after treatment must have contained the constituent in question. On the other hand, caution has to be exercised in such observations, for if other parts show no change in their staining capacity after treatment to remove a particular constituent, it need not be concluded that these parts do not contain that constituent. This is because many stains are taken up by constituents other than the one that is removed. These parts are not missed because there are other stains which do not stain the residual cell wall constituents, and by combining the observations of the results of staining of the treated and untreated sections with different stains it is possible to localise different constituents with accuracy. Oxalic acid and ammonium oxalate are only given above as examples, but sections may be submitted to various treatments (*e.g.* enzymes) according to the nature of the constituent under investigation. The value of the results obtained by differential staining will depend largely on the thoroughness with which a constituent is removed by a particular reagent and by the extent to which that reagent is specific in its action. The writer has mainly concentrated on the following treatments, with a view to locate the principal constituents which take part in metabolism. Even if the sections be treated with a reagent which is not specific it is possible to make good deductions by combining the results of staining with those of another treatment. Sections were treated by boiling with 0·5 % ammonium oxalate, 0·5 % ammonium oxalate in 3 % ammonia solution, 4 % sodium hydroxide solution, 3 % hydrochloric acid, or 95 % alcohol. This was usually carried out by heating the sections with the respective reagents in test tubes immersed in a boiling water-bath for 6–8 hours. To ensure complete extraction the supernatant liquid was decanted off every hour and the extraction continued with the fresh reagent. After the extraction was thus completed the sections were washed with hot distilled water and stained as already described.

Throughout the work the sections of the unfixed material were cut with a Leitz hand microtome or by free hand, and in the case of timber with an ordinary carpenter's plane, which gave quite good sections.

The results of observations by the above method of differential staining with sections of the leaf and stem of *Pinus sylvestris* are given in Tables IV and V.

PROBABLE CONSTITUENTS OF DIFFERENT PARTS OF THE LEAF OF *PINUS SYLVESTRIS* (Table IV).

The epidermis in the leaf of *Pinus sylvestris*, after treatment with ammonia and ammonium oxalate, shows a slight decrease in the intensity on staining with ruthenium red and haematoxylin, indicating that only traces of pectic substances are present. With eosin and haematoxylin, after treatment with sodium hydroxide, a marked change is observed indicating the presence of hemicelluloses. The negative zinc chloride-iodine reaction indicates the absence of true celluloses.

The cell wall of the hypodermal cells gives the cellulose reaction with zinc chloride-iodine, as well as the phloroglucinol reaction, and in this respect it is differentiated from lignocellulose. The cell contents of some of them are stained by alkannin showing the presence of resinous substances. The behaviour with gossypimin and haematoxylin indicates the presence of galactan, but hemicellulose and pectin are absent. The persistence of the staining with ruthenium red after various treatments suggests that the cell wall of these hypodermal cells is composed mainly of an oxidised form of cellulose, such as α-oxycellulose, which is not dissolved by dilute alkali. The contents of these cells also give a strong reaction with benzidine and hydrogen peroxide for oxidases.

Endodermal cells also give the cellulose reaction, contain oxidases, stain persistently like the hypodermal cells with ruthenium red and contain traces of aromatic substances (probably phenolic glucosides) which stain with the vanillin reagent. They are stained by phloroglucinol, especially at the junction of two cells. On treatment with concentrated sulphuric acid the endodermal ring remains intact. Parenchyma cells stain a bluish-green colour with Congo red indicating the presence of carbohydrates of acidic nature such as pectic acid; they also contain oxidases. They do not give the cellulose reaction until these are removed. Vanillin reagent gives a strong coloration in these cells. Their behaviour with eosin, ponceau, iodine green, and gossypimin after different treatments shows that they are principally composed of unoxidised celluloses, cellulose-α and -β, together with pectic substances.

Transfusion tissue like the hypodermal cells stains with phloroglucinol and zinc chloride-iodine. The contents of some cells stain with alkannin and give a strong coloration with vanillin reagent. Results of staining with ruthenium red, iodine green, borax carmin and ponceau show these cells to be composed of α-oxycellulose and neutral pectic substances.

Table IV. *Transverse sections of leaf of* Pinus sylvestris.

	Epidermis	Hypodermis	Endodermis	Parenchyma	Transfusion tissue	Xylem	Phloem	Resin duct	Secretory cells
Malachite green:									
Untreated	+4	+2	+2	–	+2	+3	–	–	+3
Ammonium oxalate and ammonium hydroxide	+4	+2	+2	–	+2	+3	–	–	+3
Sodium hydroxide	+4	+3	+3	–	+2	+4	–	–	–
Ammonium oxalate	+4	+3	+3	–	+2	+4	–	–	+3
Alcohol	+4	+2	–	–	–	+4	–	–	–
Hydrochloric acid	+3	+3	+2	–	+2	+3	–	–	–
Borax carmin:									
Untreated	–	+2	–	–	+2	–	+4	+4	+4
Ammonium oxalate and ammonium hydroxide	–	+4	–	+2	–	–	+3	+2	+4
Sodium hydroxide	+2	+3	–	+1	–	–	+4	–	–
Ammonium oxalate	–	+4	–	+2	–	–	+4	+3	+4
Alcohol	–	+3	–	+3	–	–	+4	–	+4
Hydrochloric acid	–	+3	–	+2	–	–	+4	+2	+4
Aniline blue picrate:									
Untreated	–	– (+4)*	– (+4)*	+2	–	–	+4	+2	– (+4)
Ammonium oxalate and ammonium hydroxide	+1	+1 (+4)*	–	+4	–	+4	–	–	–
Sodium hydroxide	–	+1	–	–	–	–	–	–	–
Ammonium oxalate	–	– (+4)*	–	+4	+2	–	+4	+3	– (+3)
Alcohol	–	–	–	+4	+1	–	+1	–	–
Hydrochloric acid	–	– (+4)*	–	+4	–	–	+3	+4	– (+3)
Iodine green:									
Untreated	+3	+2 (+4)*	+3	+2	+3	+4	+4	+4	+2
Ammonium oxalate and ammonium hydroxide	+2	+2	+3	–	+3	+4	–	–	–
Sodium hydroxide	+3	+3	+3	–	+3	+3	–	–	–
Ammonium oxalate	+2	– (+4)*	+3	+1	+1	+2	+1	+1	– (+1)
Alcohol	+3	+2	+2	+1	+2	+2	+3	–	– (+2)
Hydrochloric acid	+2	+2	+1	–	+1	+2	–		– (+2)
Ponceau:									
Untreated	+2	+2	+4	+4	+3	+2	+3	+3	+2
Ammonium oxalate and ammonium hydroxide	+2	+2	+3	+4	+3	+2	+3	+4	+1 (+4)
Sodium hydroxide	+1	+3	+2	+1	+3	+3	–	–	–
Ammonium oxalate	+2	+2	+3	+4	+3	+3	+3	+4	+2
Alcohol	+2	+2	+3	+4	+3	+3	+2	+2	+1
Hydrochloric acid	+2	+2	+3	+4	+3	+3	+2	+3	+2
Ruthenium red:									
Untreated	+1	+3	+3	+3	+3	+3	+4	+3	+4
Ammonium oxalate and ammonium hydroxide	+1	+3	+3	+1	+3	+3	+4	+1	+3 (+4)
Sodium hydroxide	+1	+3	+3	–	+3	+3	–	–	–
Ammonium oxalate	–	+3	+3	+1	+3	+2	+4	–	+3
Alcohol	+1	+2	+3	+3	+3	+3	+4	+3	+3
Hydrochloric acid	–	+3	+3	–	+3	+2	+3	+2	+4
Alkannin:									
Untreated	–	–	–	–	– (+3)*	–	–	+3	– (+2)*
Ammonium oxalate and ammonium hydroxide	–	–	–	–	–	–	–	–	–
Sodium hydroxide	–	–	–	–	–	–	–	–	–
Ammonium oxalate	–	–	–	–	–	–	–	+2	– (+1)*
Hydrochloric acid	–	(+3)*	–	–	– (+3)*			+3	– (+2)*
Gossypimin:									
Untreated	+4	+3	+3	+3	+3	+3	+3	+3	+3
Ammonium oxalate and ammonium hydroxide	+4	+2	+3	–	+3	+3	+3	–	–
Sodium hydroxide	+4	–	+3	–	+2	+4	+2	–	–
Ammonium oxalate	+4	+2	+2	–	+	+4	+2	–	–
Alcohol	+4	+2	+1	+1	+1	+2	+4	–	–
Hydrochloric acid	+4	+2	+2	–	+2	+3	+2	–	–

Table IV (*continued*).

	Epi-dermis	Hypo-dermis	Endo-dermis	Paren-chyma	Trans-fusion tissue	Xylem	Phloem	Resin duct	Secre-tory cells
nc chloride-iodine:									
Untreated	—	+4	+4	—	+4	—	+4	Yellow	+4
Ammonium oxalate and ammonium hydroxide	—	+4	—	+4	+4	—	+4	+3	+3
Sodium hydroxide	—	+3	—	+4	+4	—	+4	—	+4
Ammonium oxalate	—	+3	—	+4	+4	—	+4	+3	+4
Alcohol	—	+3	+4	—	+4	—	+4	+4	+4
Hydrochloric acid	—	+3	—†	+4‡	+4	—	+4	+4	+4
nillin:									
Untreated	—	—	+3	+4	+4	—	+4	+4	+3
Ammonium oxalate and ammonium hydroxide	—	—	—	—	—	—	—	—	—
Sodium hydroxide	—	—	—	—	—	—	—	—	—
Ammonium oxalate	—	—	—	+3	+1	—	+4	+3	+1
Alcohol	—	—	+3	+4	+3	—	+3	+4	+3
Hydrochloric acid	—	—	—	—	+2	—	+3	+4	+2
ematoxylin:									
Untreated	+2	+1	+3	+1	—	—	+3	+1	— (+3)*
Ammonium oxalate and ammonium hydroxide	+1	+2	+1	—	—	—	+2	—	—
Sodium hydroxide	—	+3	+2	—	+1	+2	—	—	—
Ammonium oxalate	—	+1	+3	+2	+1	+1	+2	—	—
Alcohol	+2	+2	+3	+2	+3	—	+3	—	+2 (+3)*
Hydrochloric acid	—	+2	+3	—	+2	+1	+3	+1	+2
rric thiocyanate:									
Untreated	—	—	—	—	—	—			
Ammonium oxalate and ammonium hydroxide	—	—	—	—	—	—	—	—	—
Sodium hydroxide	—	—	—	—	—	—	—	—	—
Ammonium oxalate	—	—	—	—	—	—	—	—	—
Alcohol	—	—	—	—	—	—	—	—	—
Hydrochloric acid	—	—	—	—	—	—	—	—	—
ngo red:									
Untreated	—	+1	+2	+1§	+4	+2	+4	+2	+4¶
Ammonium oxalate and ammonium hydroxide	+1	+1 (+3)*	+2	+1§	+3	+2	+4	+2	+3¶
Sodium hydroxide	+2	+2	+3	—	+3	+3	+4	—	+2¶
Ammonium oxalate	—	+2 (blue)	+3	+1	—	+2	+4	+2	+4¶
Alcohol	—	+1	+3	+1	+3	+1	+4	+2	+4¶
Hydrochloric acid	—	+1	+2	+2	+1	+1	+4	+3	+4¶
osin:									
Untreated	+3	—	—	+3	—	—	—	+½	—
Ammonium oxalate and ammonium hydroxide	+3	—	—	—	—	+2	—	—	—
Sodium hydroxide	—	—	—	—	—	—	—	—	—
Ammonium oxalate	+3	—	—	—	—	+3	—	—	—
Alcohol	+3	+1	+3	+4	+3	+4	+2	+3	—
Hydrochloric acid	—	—	+3	+4	—	—	+3	+3	—
hloroglucinol:									
Untreated	—	+4	+4	—	+3	+4	—	—	—
Ammonium oxalate and ammonium hydroxide	—	+2 (m.l.)	+1	—	—	+3	—	—	—
Sodium hydroxide	—	—	—	—	—	+½	—	—	—
Ammonium oxalate	—	+3 (m.l.)	+3	—	+3	+3	—	—	—
Alcohol	—	+4	+4	—	+3	+4	—	—	—
Hydrochloric acid	—	+4	+4	—	+3	+4	—	—	—

* Cell content. † Yellow. ‡ Very dark. § Bluish green. ¶ Bluish black.

— Negative. +1, +2, +3, +4 show increasing intensity of colour, +4 being the strongest.

Table V. *Sections of green stems of* Pinus sylvestris.

	Middle lamella (contents)		Middle lamella (binding layers)		Cell wall		Cell contents (remains)		Autumn wood		Spring and summer wood		Bordered pits		Pit (outer ring)		Pit partition		Medullary ray (contents)		Zone surrounding medullary ray		Resin canal		Primary xylem (remains)
	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	A.	C.	
Malachite green:																									
Untreated	+4	+4	−	−	+4	+4	×	×	+4	+4	+4	+4	+2	×	+2	×	+1	×	+4	+4	×	×	+4	+4	+4
Ammonium oxalate and ammonium hydroxide	+4	+4	−	−	+3	+4	+3	×	+4	+4	+2	+2	×	×	×	×	+1	×	+3	+3	×	×	+4	+4	+3
Sodium hydroxide	+4	+4	−	−	+3	+4	+3	×	+4	+4	+2	+2	+2	×	+2	×	+1	×	+3	+4	×	×	+4	+4	+4
Ammonium oxalate	−	−	−	−	+3	+3	+2	×	+4	+4	+3	+2	×	×	+2	×	+1	×	+3	+3	×	×	+3	+4	+3
Alcohol	−	+4	−	−	+3	+3	+2	×	+3	+3	+3	+2	+2	×	+2	×	+1	×	+2	+4	×	×	+4	+4	+3
Borax carmin:																									
Untreated	−	−	−	−	−	−	−	−	−	−	−	−	−	×	−	×	−	×	+4	+4	+3	×	+2	+3	+3
Ammonium oxalate and ammonium hydroxide	−	−	−	−	−	−	+3	+2	−	−	−	−	×	×	×	×	×	×	+4	+4	+3	×	+3	+3	+4
Sodium hydroxide	−	−	−	−	−	−	−	−	+1	+½	−	−	−	×	−	×	−	×	−	−	−	×	+3	+3	+1 +½
Ammonium oxalate	−	−	−	−	−	−	+2	+1	−	−	−	−	−	×	−	×	−	×	+4	+4	×	×	+2	+3	+4
Alcohol	−	−	−	−	−	−	+1	−	−	−	−	−	−	×	−	×	−	×	+2	+4	×	×	+3	+3	+4
Ruthenium red:																									
Untreated	+4	×	+2	+2	+½	+½	−	−	+3	+3	+½	+½	+4	×	+2	×	+4	×	+4	+4	+1	×	+4	+4	+3
Ammonium oxalate and ammonium hydroxide	−	−	−	−	+1	+1	+1	+1	+3	+3	+1	+1	−	−	−	×	−	×	+3	+3	−	−	+4	+4	+4
Sodium hydroxide	−	−	−	−	+2	+2	+2	+2	+3	+3	+2	+2	−	−	−	×	−	×	−	−	−	×	−	−	+4
Ammonium oxalate	−	+2	−	−	+1	+1	+1	+1	+3	+3	+1	+1	−	×	−	×	−	×	+4	+4	−	×	+3	+3	+3
Alcohol	−	−	−	−	−	−			+3	+3	−	−							+3	+3	−	×			+4
Aniline blue picrate:																									
Untreated	−	−	−	−	+½	+½	+3	+3	+½	+1	+½	+½	+2	×	+2	×	−	×	+4	+4	−	×	−	−	+4
Ammonium oxalate and ammonium hydroxide	×	×	×	×	+4	+4	+3	+3	+4	+4	+4	+4	×	×	×	×	−	×	+4	+4	−	×	−	−	+4
Sodium hydroxide	−	−	−	−	+3	+3	+2	+2	+4	+4	+3	+3	+4	×	+3	×	−	−	+2	+2	−	×	+1	+1	+2
Ammonium oxalate	−	−	−	−	+½	+½	+1	+1	+½	+½	+½	+½	+1	×	+1	×	−	−	+4	+4	−	×	−	−	+4
Alcohol	−	−	−	−	−	−	+1	+1	−	−	−	−	−	−	−	×	−	×	+3	+3	−	×	×	×	+4
Iodine green:																									
Untreated	−	+4	−	−	+4	+4	+4	+4	+4	+4	+3	+3	+1	×	+2	−	−	×	+4	+4	−	×	+4	+4	+4
Ammonium oxalate and ammonium hydroxide	−	+4	−	−	+4	+4	+3	+3	+4	+4	+4	+4	+1	×	+1	×	−	×	+4	+4	−	×	+4	+4	+4
Sodium hydroxide		+4	−	−	+2	+2	+1	+1	+3	+3	+2	+2	−	×	−	×	−	×	+1	+1	−	×	+3	+3	
Ammonium oxalate	−	+4	−	−	+4	+4	+2	+2	+4	+4	+3	+4	+2	×	+1	×	−	−	+4	+4	−	×	+3	+3	+4
Alcohol	−	+4	−	−	+4	+4	+2	+2	+4	+4	+3	+3	+1	×	+1	×	−	−	+4	+4	−	×	+4	+4	+4
Ponceau:																									
Untreated	−	−	−	−	+½	+½	−	−	+½	+½	+½	+½	−	×	−	×	−	×	+4	+4	−	×	+3	+3	+4
Ammonium oxalate and ammonium hydroxide	−	−	−	−	+2	+2	+2	−	+3	+3	+2	+2	−	×	×	×	×	×	+4	+4	−	×	+4	+4	+4
Sodium hydroxide	−	−	−	−	+½	+½	−	−	+1	+1	+½	+½	−	×	−	×	−	×	+4	+4	−	×	−	−	+4
Ammonium oxalate	−	−	−	−	+½	+½	−	−	+1	+1	+½	+½	−	×	−	×	−	×	+4	+4	−	×	+3	+3	+4
Alcohol	−	−	−	−	+½	+½			+½	+½	+½	+½	−	×	−	×	−	×	+2	+2	+1	×	+3	+3	+4

Haematoxylin:																										
Untreated	—	—	+3	—	—	—	+2	+2	—	—	—	—	+½	×	+½	×	—	×	+2	+2	—	×	—	—	+1	
Ammonium oxalate and ammonium hydroxide	—	—	—	—	+1	+1	+2	+2	+2	+2	+1	+1	—	×	—	×	—	×	+3	+3	—	×	+2	+2	+3	
Sodium hydroxide	—	—	+3	+3	+4	+4	+3	+3	+4	+4	+4	+4	+1	×	+1	×	—	×	+3	+3	—	×	+4	+4	+4	
Ammonium oxalate	—	—	+1	—	+2	+2	+3	+3	+2	+2	+3	+3	+4 centre	×	+3	×	—	×	+4	+4	—	×	+3	+3	+3	
Alcohol	—	—	+2	—	+2	+2	+3	+3	+2	+2	+3	+3	+4 centre	×	+2	×	—	×	+3	+3	—	×	+3	+3	+2	
Ferric thiocyanate:																										
Untreated	—	—	—	—	—	—	—	—	—	—	—	—	—	×	—	×	—	×	+1	+1	—	×	+1	+1	+1	
Ammonium oxalate and ammonium hydroxide	—	—	—	—	+½	+½	—	—	+1	+1	+½	+½	—	×	—	×	—	×	+2	+2	—	×	+2	+2	+2	
Sodium hydroxide	—	—	—	—	—	—	—	—	+½	+½	—	—	—	×	—	×	—	×	—	—	—	×	—	—	+1	
Ammonium oxalate	—	—	—	—	—	—	—	—	—	—	—	—	—	×	—	×	—	×	+1	+1	—	×	+2	+2	+2	
Alcohol	—	—	—	—	—	—	—	—	+½	+½	—	—	—	×	—	×	—	×	+2	+2	—	×	+½	+½	+2	
Congo red:																										
Untreated	—	—	—	—	—	—	+3	+3	+½	+½	—	—	+1	×	+1	×	—	×	+4	+4	+3	×	+2	+2	+1	
Ammonium oxalate and ammonium hydroxide	—	—	—	—	+½	+½	+3	+3	+1	+1	+½	+½	+2	×	+2	×	—	×	+4	+4	+3	×	+3	+3	+4	
Sodium hydroxide	—	—	—	—	+½	+½	+2	+2	+1	+1	+½	+½	+1	×	+1	×	—	×	+1	+1	—	×	+3	+3	+2	
Ammonium oxalate	—	—	—	—	+½	+½	+2	+2	+1	+1	+1	+1	+1	×	+1	×	—	×	+4	+4	—	×	+4	+4	+3	
Alcohol	—	—	—	—	—	—	+1	+1	—	—	—	—	+½	×	+½	×	—	×	+3	+3	—	×	+2	+2	+3	
Eosin:																										
Untreated	—	—	—	—	+3	+3	+1	+1	+3	+3	+3	+3	+1	×	+1	×	—	×	+3	+3	—	×	+4	+4	+3	
Ammonium oxalate and ammonium hydroxide	—	—	—	—	+3	+3	+1	+1	+3	+3	+3	+3	+1	×	+1	×	—	×	+3	+3	—	×	+3	+3	+4	
Sodium hydroxide	—	—	—	—	+1	+1	+1	+1	+2	+2	+1	+1	+½	×	+½	×	—	×	+1	+1	—	×	+3	+3	+1	
Ammonium oxalate	—	—	—	—	+1	+1	+1	+1	+3	+3	+2	+2	+½	×	+½	×	—	×	+3	+3	—	×	+4	+4	+3	
Alcohol	—	—	—	—	+2	+2	+2	+2	+3	+3	+2	+2	+1	×	+1	×	—	×	+3	+3	—	×	+3	+3	+4	
Phloroglucinol:																										
Untreated	—	—	+2	+2	+3	+3	—	—	+4	+4	+2	+2	—	—	—	—	—	×	—	—	—	×	+4	+4	+2	
Ammonium oxalate and ammonium hydroxide	—	—	—	—	—	—	—	—	+1	+1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	
Sodium hydroxide	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ammonium oxalate	—	—	—	—	+3	+3	—	—	+3	+3	+2	+2	—	—	—	—	—	—	—	—	—	—	+3	+3	+3	
Alcohol	—	—	—	—	+½	+½	—	—	+½	+½	+½	+½	—	—	— centre	—	—	—	—	—	—	—	+½	+½	+½ pith	
Alkannin:																										
Untreated	+3 in places	+3	—	—	—	—	—	—	—	—	—	—	—	—	+3	+3	—	—	+3	+3	—	—	+3	+3	+4	+4
Ammonium oxalate and ammonium hydroxide	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Sodium hydroxide	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Ammonium oxalate	+3	+3	—	—	—	—	—	—	—	—	—	—	—	—	+3	+3	—	—	+3	+3	—	—	+4	+4	+4	+4
Alcohol																										

A, Longitudinal section. C, Transverse section. — Negative. +1, +2, +3, +4 show increasing intensity of colour, +4 being the strongest. × Not observed.

Xylem cells give a strong phloroglucinol reaction like the true lignocelluloses and do not give the colour for cellulose with zinc chloride-iodine. They do not contain oxidases, and their general behaviour with different stains suggests the presence of hemicelluloses, pectic substances and α-oxycellulose.

Phloem does not give a coloration with phloroglucinol but gives a strong coloration with the vanillin reagent for aromatic substances and with zinc chloride-iodine for cellulose. It is also very active in oxidases. Its staining reactions after treatment with sodium hydroxide indicate the presence of hemicellulose but not of pectic substances.

The resin canal does not give directly the iodine reaction for cellulose until the pectic substances of which it is principally composed are previously removed. The presence of pectic substances is confirmed by its behaviour with eosin, ruthenium red, gossypimin, etc. It is not stained by phloroglucinol, but it gives a deep coloration with the vanillin reagent for aromatic substances. It is also stained by alkannin and gives a strong reaction for oxidases. Its behaviour with borax carmin and Congo red also indicates the presence in small amounts of hemicellulose.

Secretory cells do not stain with phloroglucinol; they give a reaction for cellulose direct, and their contents are stained with alkannin and vanillin reagents. They also give a reaction for oxidases. Their behaviour with ruthenium red, ponceau and borax carmin, shows that these cells are composed principally of cellulose together with hemicelluloses but they do not contain pectic substances. Congo red stains these cells bluish red, indicating that the secretion of these cells is of an acidic character.

PROBABLE CONSTITUENTS OF DIFFERENT PARTS OF THE STEM OF *PINUS SYLVESTRIS* (Table V)

The middle lamella of many plant tissues can be differentiated with certain stains into three layers, a thin central layer which is bounded on either side with another layer which sometimes stains differently from the central one. Hence the results of staining the middle lamella are given separately in two columns. In the first column are given the results of staining of the central layer (contents). This layer is stained by alkannin and also by the vanillin reagent, thus indicating the presence of resinous substances. It is deeply stained in young stems by ruthenium red but not after treatment with ammonium oxalate, which shows that it is composed of neutral pectic substances. It is not stained by phloroglucinol. The binding layers are not well developed in stems 10–12 years old. Generally, the thin central layer is well marked in the xylem cells close to the cambium layer. Proceeding towards the centre of the stem the central layer almost disappears and is not stained by the majority of colours. In older stems the middle lamella is more strongly differentiated.

The cell wall of the secondary xylem stains deeply with phloroglucinol but curiously it is not stained after the sections are extracted with alcohol. The staining reactions with eosin, iodine green, and ponceau suggest the presence of hemicellulose and of traces of pectin. It is not stained by Congo red and zinc chloride-iodine, but in transverse section a faint blue colour is observed in the layer binding the cell cavity. This suggests that these cell walls are composed of lignocellulose, together with some hemicellulose and a thin coating towards the centre of the cell cavity of free cellulose. Some differences are observed between the autumn and the spring wood. The autumn wood always seems to stain more deeply than the spring wood. Its behaviour with ruthenium red suggests the presence of α-oxycellulose which seems to be absent from the spring wood.

A number of theories have been advanced to explain the difference between the spring and the autumn wood, which lies in the decrease in the diameter of the tracheides and the increase in the thickness of their walls. The writer is of the opinion that the relatively greater thickness of the cell wall in autumn wood may be the result of an increase in the concentration of the nutritive material. On determining the influence of concentration of the nutritive substances on the thickness of the cell wall of yeast it is found that the thickness of the cell wall increases with the concentration of the medium.

Cell remains are stained by zinc chloride-iodine, but not with phloroglucinol. Their staining reactions with ruthenium red, aniline blue picrate, iodine green and Congo red, indicate the absence of pectin and hemicellulose and they are probably composed exclusively of hydrocellulose.

The bordered pit is stained in some respects like the middle lamella by ruthenium red and alkannin.

The medullary ray is stained by alkannin and zinc chloride-iodine, and its staining reactions with ruthenium red, eosin, borax carmin, ferric thiocyanate and Congo red indicate the presence of hemicellulose, pectins and gums. A strong coloration is observed in these cells with the vanillin reagent.

Reactions of the resin canal after different treatments with stains such as alkannin, ponceau and ferric thiocyanate, strongly indicate the presence of hemicellulose, gums and lignin or resin. The presence of lignin or resin is further confirmed with the vanillin reagent.

Primary xylem remains stain deeply with ruthenium red and appear to contain oxycellulose. They are also stained by zinc chloride-iodine but feebly by phloroglucinol.

Oxidases are distributed in all those parts through which migration of food materials is taking place. They differ from the oxidases of the leaf in that they give the blue coloration with guaiacum.

The middle lamella in timber appears to be of different composition from that of the young wood. Pectin substances are absent from the central layer and they are replaced at a later stage by lignin, gums and hemicellulose as may be judged from its reactions with borax carmin, malachite green and ferric thiocyanate. It is also stained by phloroglucinol and vanillin reagent. Pectin is similarly absent from the binding layers of the middle lamella in timber, and their reactions with stains such as haematoxylin, ferric thiocyanate, Congo red and eosin, show them to be composed of hemicellulose and gums.

Attention may be drawn to the results of staining with some dyes after extraction with alcohol. In some parts after extraction the results are altogether negative. It is probable that traces of resinous or aromatic substances are dispersed over certain plant cells, which are removed by alcohol. This is a matter of importance since alcohol is often used as a fixative and as a preserving medium.

These attempts at differentiating the various constituents of plant tissues by differential staining and microscopical examination are sufficient to show the limitations and the difficulties which are to be confronted in histological work.

SUMMARY.

An attempt has been made to unravel the microchemical structure of plant tissues, and to interpret in chemical terminology the distribution of the different units comprising the cell.

Cellulose-α, -β and -γ, oxycelluloses-α, -β and -γ, hydrocellulose, mannan, galactan, pectin, amylohemicellulose, hemicellulose, gums, starch, lichenin and chitin have been isolated in the purest possible condition and their staining capacities determined.

The relative amounts of the different cellulosic constituents vary widely according to the cultural conditions, general environment and the nature of the plant. Cellulose-β and -γ show a greater affinity for stains than cellulose-α. Oxycelluloses, unlike cellulose-α, -β and -γ and hydrocellulose, are stained by ruthenium red.

Two kinds of hydrocellulose are found which can be differentiated by the coloration given by one of them with iodine in potassium iodide solution.

The microchemical structure of the leaf and stem of pine is investigated by the removal of specific constituents by suitable chemical treatment with solvents such as ammonia, ammonium oxalate, sodium hydroxide, hydrochloric acid and alcohol, followed by differential stainings.

Treatment with ammonium oxalate shows that the middle lamella in young stems of pine is composed of neutral pectic substance whilst in old wood (timber) it is composed of lignin, gums and hemicellulose, pectin being absent.

Extraction of the tissues with alcohol gives inconsistent results.

Resins react in some respects like lignin and are associated with gums in different parts of the same plant.

Lignification sets in very early in the life history of the plant and occurs in ground tissue, fibro-vascular bundles, medulla and cuticularised cells.

The writer in conclusion feels it a great pleasure to express her sincere gratitude to Prof. Arthur R. Ling and Dr Dinshaw R. Nanji for their valuable criticism and for their continuous encouragement and interest in the course of these investigations. Her thanks are also due to Mr J. Harris for supplying her with specimens of different timbers.

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CXLII. THE EFFECT OF SHORT PERIODS OF COLD STORAGE ON BEEF AND MUTTON.

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This work was started at the request of Dr F. E. Wynne, Medical Officer of Health for Sheffield, who wished to construct a cold room for storage of carcases in connection with proposed new public abattoirs.

It was stated by local butchers that meat is "spoilt" by being placed in a cold room for any period of time, however short, and that it was necessary to take carcases straight from slaughter to the butchers' shops. No experiments appear to have been made in this field of research except some on the autolysis of beef and mutton by Fearon and Foster [1922] and by Callow [1925] on the freezing of cod and its effect on autolytic change. These workers used antiseptics in their experiments, and therefore statements with regard to preserving meat for food cannot be based on their results. Work on the carnosine content of beef kept at 25° F. by Clifford [1922] showed a fall in this extractive, which, however, did not take place for several weeks, and would not affect the question in point.

The experiments to be described in this paper were carried out to show the effect, if any, on beef and mutton kept in cold storage for periods up to 13 days, and duplicate estimations of the total nitrogen, soluble nitrogen, amino nitrogen, carnosine and creatine were made at approximately 3 day intervals.

Experimental Methods.

The meat was obtained on the day of slaughter and sent straight to the National Institute for Medical Research at Hampstead, where, by the kind permission of Dr Dale, it was placed in the cold rooms. For the experiments on mutton, a leg was taken and cut in half across the muscles. Half was then put into the inner room at a temperature of 25° F., and the other half in the outer room at a temperature of 35° F. Evaporation was avoided by suspending the joints in large glass filtrate jars, covered with clock glasses. At intervals, samples of muscle weighing 200–300 g. were cut off the joints and put into separate thermos flasks, previously left unstoppered in the chambers for 5 to 6 minutes to attain the same temperature as the room where the meat was kept. These flasks were then corked, brought to the laboratory, and samples weighed as rapidly as possible for the various estimations which were always duplicated. Whenever possible the same muscle was used from both halves of the leg, so that the results from meat kept at 35° F. and at 25° F. are strictly comparable.

With beef exactly the same method was adopted, but in Exp. 1 meat from the shin was used, and in Exp. 2 a thick joint of fillet steak.

Total nitrogen. This was estimated by Kjeldahl's method as described by Cole [1920], about 1–2 g. meat being taken. Care was exercised to see that pieces from the same muscle were used for this and all other estimations on the same day, and when possible throughout the whole experiment. However, on one or two occasions it was difficult to identify the special muscles in a piece of frozen meat cut to a suitable size to go into the neck of a thermos flask.

From the tables of Exps. 1 and 2 it will be seen that there is a slight rise in total nitrogen suggesting that some water is lost by evaporation when beef is kept under the experimental conditions described; this loss, however, is slight. With Exps. 3 and 4 on mutton no such rise was seen and evaporation must have been negligible.

Soluble nitrogen. For estimations of non-protein soluble nitrogen the following method was used. 5–10 g. muscle were weighed and cut up very finely with scissors on a watch glass. This mince was washed into a beaker with about 100 cc. distilled water and covered with a watch glass. The beaker was then heated slowly till the water just came to the boil. The liquid was then poured off the meat, and a second boiling with 50 cc. distilled water carried out in a similar way. The first extract was then added to the meat and second extract, and 5 cc. of 20 % metaphosphoric acid added to precipitate the protein. The mixture was allowed to stand overnight and was then filtered into a 250 cc. measuring flask, the residue was well washed with distilled water, the washings being allowed to run into the flask, and finally, the liquid was made up to the mark with distilled water. Kjeldahl estimations were then carried out on 50 cc. portions of this filtrate to give the amount of soluble nitrogen present.

In the results shown in the tables, besides the actual experimental figures obtained, the ratio of soluble nitrogen to total nitrogen is given, since this is the means by which Fearon and Foster [1922] and Callow [1925] measure the rate of autolytic change. It will be seen that no change takes place in the ratio in short periods of time with beef at 35° F. or 25° F. In Exp. 3 on mutton the same is true, but in Exp. 4 there is a slight rise throughout the experiment showing a very small amount of autolysis at 25° F. in 10 days.

Amino nitrogen. The amino nitrogen content of the meat was determined on portions of the same filtrate employed for the estimation of soluble nitrogen. Van Slyke's apparatus was used.

It can be seen from the tables that there was no rise or fall in the amino nitrogen content of beef or mutton during the experimental period.

Carnosine. Estimations of this extractive were made according to a method described by Clifford [1921]. Again the estimations were carried out on portions of the filtrate prepared as stated for soluble nitrogen. Carnosine remained constant except in one case, that of Exp. 2 on beef, kept at 35° F., when on the sixth day there was a marked fall. This was probably due to an incipient putrefaction, as on the eighth day the meat was definitely decomposed.

Creatine and Creatinine. For the estimation of creatine 1–1·5 g. muscle was weighed into a flask, 20 cc. of 3 % hydrochloric acid were added, the flask plugged with cotton wool, and the whole autoclaved at 130–140° for 30 minutes. The creatinine thus formed from the muscle creatine together with any preformed creatinine present was estimated by Folin's method as described by Plimmer [1920].

There was no change in this extractive during the experimental periods either at 35° F. or at 25° F.

Experimental Results.

The results of the experiments are given in the following tables:

Exp. 1. *Shin of beef.*

	G. per 100 g. meat			G. per 100 g. meat			
Day	Total N	Sol. N	Sol. N / Total N %	Amino N	Carnosine	Creatine expressed as creatinine	
0	A. 2·82	0·349	12·02	0·731	0·980	0·359	
	B. 3·10	0·376	12·11	0·765	0·976	0·370	
3	A. 2·84	0·360	12·7	0·760	0·981	0·378	
	B. 2·81	0·359	12·8	0·737	0·980	0·375	B exactly same in appearance as A
6	A. 3·33	0·420	12·3	0·764	0·980	0·362	Large spicules of ice in frozen meat. Copious drip when thawed. Outer room meat very sticky and smells slightly musty
	B. 3·38	0·422	12·5	0·751	0·979	0·370	
8	B. 3·58	0·458	12·5	0·764	0·983	0·411	Meat in outer room definitely bad, therefore Exp. A stopped. Copious drip on thawing B meat
10	B. 3·56	0·438	12·3	0·760	0·980	0·370	,, ,,
13	B. 3·56	0·436	12·2	0·746	0·984	0·365	,, ,,

A = outer room 35° F. B = inner room 25° F.

Discussion.

These experiments were carried out in July 1925 when the weather was exceptionally hot and sultry with frequent thunder-storms, and therefore in all probability represent the maximum *post-mortem* change. The weather conditions were rather worse in Exps. 2 and 4 than in 1 and 3.

With such atmospheric conditions it appears impossible to keep meat in a condition fit for human food at a temperature slightly above freezing point (35° F.) for longer than 3–4 days. This is shown by the fact that on the sixth day of these experiments there was a decidedly musty smell, and an appearance of incipient decomposition in both beef and mutton, and on the eighth day putrefaction had definitely started in three out of the four experiments. It is true that in these experiments the meat was shut up in glass jars, and therefore did not have the benefit of air currents, but, to balance this, in a cold room connected with an abattoir whole or half carcases would be stored instead of comparatively thin pieces of muscle and consequently they would not cool so rapidly.

Exp. 2. *Beef (fillet steak).*

	G. per 100 g. meat			G. per 100 g. meat			
Day	Total N	Sol. N	Sol. N / Total N %	Amino N	Carnosine	Creatine expressed as creatinine	
0	3·44	0·397	11·2	0·915	0·981	0·450	Day of slaughter
	B. 3·50	0·404	11·2	0·897	0·980	0·456	
3	A. 3·64	0·403	11·5	0·820	0·979	0·413	
	B. 3·66	0·421	11·1	0·890	0·981	0·452	
6	A. 3·77	0·423	11·2	0·835	0·814*	0·452	A with faint musty smell and sticky. Spicules of ice in B very red and dripping on thawing * Fall probably due to incipient decomposition
	B. 3·86	0·439	11·3	0·847	0·990	0·452	
8	B. 3·67	0·430	11·8	0·947	0·982	0·409	Meat in outer room quite bad; experiment therewith abandoned. Very copious drip on thawing B
	B′. 3·66	0·403	11·2	0·976	0·987	0·430	
10	B. 3·58	0·413	11·5	0·908	0·987	0·419	„ „
	B′. 3·57	0·425	11·3	0·909	0·980	0·425	

A = outer room 35° F. B = inner room 25° F.

Exp. 3. *Leg of mutton.*

	G. per 100 g. meat			G. per 100 g. meat			
Day	Total N	Sol. N	Sol. N / Total N %	Amino N	Carnosine	Creatine expressed as creatinine	
0	3·80	0·478	12·2	0·759	0·338	0·460	Day of slaughter
	—	0·469	—	0·784	0·332	0·465	
3	A. 3·58	0·444	12·4	0·848	0·341	0·476	
	B. 3·53	0·448	12·7	0·858	0·336	0·455	
6	A. 3·64	0·454	12·4	0·872	0·344	0·433	Frozen meat with ice spicules. Very slight musty smell in A
	A. 3·80	0·473	12·4	0·826	0·346	—	
	B. 3·76	0·462	12·3	0·862	0·339	0·416	
8	A. 3·76	0·473	12·3	0·870	0·340	0·468	Slight musty smell in A and meat sticky * Some NH_3 lost
	B. 3·81	0·457*	12·1	0·865	0·335	0·455	
10	A. 3·68	0·451	12·3	0·928	0·331	0·478	Meat more sticky and smell stronger in A
	B. 3·63	0·446	12·3	0·919	0·330	0·465	
13	A. 3·38	0·411	12·2	0·886	0·320	0·470	„ „
	B. 3·18	0·361	11·3	0·860	0·320	0·465	

A = outer room 35° F. B = inner room 25° F.

Exp. 4. *Leg of mutton.*

	G. per 100 g. meat		Sol. N / Total N	G. per 100 g. meat			
Day	Total N	Sol. N	%	Amino N	Carnosine	Creatine expressed as creatinine	
0	3·52	0·394	11·2	0·815	0·323	0·495	Day of slaughter
	3·47	0·390	11·2	0·814	0·338	—	
3	A. 3·37	0·415	12·3	0·827	0·320	0·491	
	B. 3·45	0·484	14·1	0·799	0·328	0·540	
6	A. 3·31	0·423	12·7	0·744	0·306	0·540	Slight musty smell in A. Spicules of ice in B. B very wet on thawing
	B. 3·43	0·414	12·1	0·823	0·330	0·499	
8	B. 3·55	0·448	12·3	0·783	0·330	0·452	A quite bad; therefore abandoned and two muscles in B used. Very copious drip on thawing of B
	B′. 3·20	0·405	12·6	0·821	0·325	0·462	
10	B. 3·19	0·444	13·9	0·788	0·328	0·311*	*Some drip lost
	B′. 3·28	0·445	13·2	0·767	0·331	0·488	

A = outer room 35° F. B = inner room 25° F.

This decomposition of freshly killed meat kept at 35° F. is superficially surprising. That this happens whenever the outside weather conditions are hot and sultry is borne out by the experience of the engineer in charge of the chilling rooms at the Islington Cattle Market. He states that in hot English weather it is impossible to keep carcases in good condition at 35° F. for more than a few days though with cool outside conditions they can be kept for weeks at that temperature. For any prolonged period of storage during the hot weather in England the temperature must be below freezing point. This difference in the storage properties of a room at a constant temperature of 35° F. according to varying outside atmospheric conditions may possibly be due to one or both of two reasons. The first is that in hot weather *post-mortem* changes take place more rapidly in the meat during the period between slaughter and the time when it attains the temperature of the chilling room, than is the case in cold weather. The second explanation is that the outside air drawn into the freezing chamber is more heavily laden with bacteria in hot weather than in cold, and consequently the organisms are able to decompose the meat by sheer numbers, even at a temperature where bacterial action is necessarily retarded. If this is so, the storage properties of a chilling room should be greatly increased by some method of filtering the air before it is allowed to enter the storage chamber.

The likelihood of there being no change in the composition of meat stored at 35° F. given cool outside conditions is supported by Exp. 3 on mutton where comparatively slight changes occurred, viz. a musty odour from the eighth to the thirteenth days and a sticky condition of the muscle without any definite and unmistakable putrefaction. Chemical estimations carried out on this meat showed no measurable change in composition.

Meat stored below freezing point (25° F.) kept indefinitely as would be expected, and no change in the appearance of the meat could be seen up to the third day of storage. To the naked eye it looked like fresh meat, or meat chilled to 35° F. By the sixth day, however, the meat at 25° F. was full of ice spicules, and had turned the characteristic bright red colour of frozen meat. There was also a copious drip on thawing this "iced" meat.

Both the frozen and chilled meats show a little surface sweating on being brought into warm air even as early as the third day of storage, but this is purely the result of bringing a cold object into a warm humid atmosphere.

The changes in frozen meat up to 13 days appear to be physical, since no chemical alteration could be detected in the total nitrogen, soluble nitrogen, ratio of soluble nitrogen to total nitrogen, amino nitrogen, carnosine or creatine contents of beef or mutton. The "drip" of frozen meat is probably due to the rupture of the muscle cells by ice spicules.

SUMMARY.

1. Beef or mutton kept at 25° F. and 35° F. appear identical in appearance with freshly killed meat up to the third day of storage.

2. Beef and mutton kept at 25° F. show ice spicules and the red colour characteristic of frozen meat on the sixth day of storage.

3. In hot English weather beef and mutton will not keep for 6 days in a room at 35° F.

4. There is no change in total nitrogen, soluble nitrogen, amino nitrogen, carnosine or creatine in meat kept at 35° F. for 3 days, or at 25° F. for 13 days.

Thanks are due to Dr Dale for kindly allowing the meat to be placed in the cold rooms at the Medical Research Institute at Hampstead.

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CXLIII. ANALYSIS OF PROTEINS.

V. VAN SLYKE'S METHOD OF DETERMINATION OF NITROGEN DISTRIBUTION.

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The method of analysis of proteins devised by Van Slyke [1911] has been used by one of us since its first description. Reference has already been made to the difficulty experienced in securing a series of consecutive results agreeing closely with one another. In the search for the cause, the estimation of arginine and the amino nitrogen of the hexone bases was studied by Plimmer [1916, 1924]. A series of analyses of gelatin showing the erratic results obtained was published by Plimmer and Shimamura [1924], and Rosedale [1922] experienced similar discrepancies with analyses of the proteins of flesh. In all the experiments the discordant results appeared to arise in connection with the precipitation and treatment of the phosphotungstates of the hexone bases.

Although this method has been largely used by workers in America, they do not specially mention discrepancies such as we have observed, but reference to the published data in many cases reveals large differences in the results of the experiments. Similar difficulties have evidently been observed, since it has been proposed by the American Association of Official Agricultural Chemists [1924] to form a committee to investigate the method with a view of standardising the technique.

Van Slyke's method of protein analysis presents so many advantages that it has seemed to us desirable to study the details in order to ascertain at what stage the discrepancies occur. A long series of comparative analyses of gelatin, haemoglobin and caseinogen have been made by us independently and simultaneously during the past three years with chief reference to the treatment of the phosphotungstate precipitate. The experience thus gained has led to the adoption of simpler apparatus for the amide estimation and the omission of the long processes of evaporation. The data are thus more rapidly obtained and possible sources of error eliminated. The proteins used in this investigation were chosen for variety of content of hexone bases—gelatin with high arginine and little histidine, haemoglobin with high histidine and caseinogen with each of the three bases in moderate amount. Cystine is not present in these proteins except in very small amounts and its presence has been neglected in these experiments. Its estimation by this method is postponed for future work.

Van Slyke's method of determining nitrogen distribution in proteins has been investigated by Gortner and Sandstrom [1925], whose results have appeared since the completion of our experiments.

Experimental.

The three proteins used in these studies were Coignet's gold label gelatin, an old sample of caseinogen from the Rhenania company of Aix-la-Chapelle, and a sample of crystallised horse haemoglobin prepared several years ago; it had lost its crystalline form and had become insoluble and brown in colour.

In order to carry out the comparative experiments designed for these investigations, it was necessary to use a considerable quantity of each of the three proteins. Amounts varying from 50 to 300 g. were hydrolysed by boiling under a reflux condenser for 36 hours with 5 parts of 25 % hydrochloric acid. The hydrolysed solutions were evaporated *in vacuo* to a thick paste, the paste dissolved in water and the water again evaporated off *in vacuo*, so as to remove hydrochloric acid as far as possible. The residue was dissolved in water and the volume made up to 2000 or 6000 cc., so as to contain from 0·3 to 0·4 g. of nitrogen per 100 cc. representing about 2·5 to 3 g. of protein. From 10 to 20 or more analyses could thus be made with 100 cc. portions of the same solution. The solutions were preserved in the earlier experiments with toluene, in the later ones with thymol. The latter was preferable, since the layer of toluene made exact measurements of 100 cc. portions troublesome. Complete analyses of the distribution of nitrogen were made in all experiments, but attention was chiefly given to the estimation of amide and humin nitrogen and the treatment of the precipitate of the phosphotungstates. For convenience of reference and for comparison, all the data are given in a series of tables (pp. 1009–1011). These data are from the results by one of us. The data by the other show exactly the same differences and are omitted to save the extra printing.

Amide and Humin Estimation.

According to Van Slyke, the amide estimation is made by adding an excess of a 10 % suspension of milk of lime to a 100 cc. portion, and, with the addition of alcohol to prevent frothing, distilling off the ammonia *in vacuo* and collecting it in excess of standard acid contained in two distilling flasks connected together to act as receiver.

As this process of collecting ammonia was cumbersome, and, in view of the work of Folin on estimating ammonia by aeration, unnecessary, it was modified to resemble the Folin technique. The side piece of the double necked distilling flask was bent at right angles so that it could be attached to a short double surface condenser set up in a filter flask. A Folin tube and bulb pierced with small holes was fastened by rubber tubing to the end of the condenser and reached to the bottom of the filter flask. It was found advisable to use a large filter flask of 1500 to 2000 cc. capacity, so that the bulb could be well covered with standard acid and water and so as to prevent splashing.

Distillation is thus very conveniently carried out, and, if required, two sets of apparatus can be set up side by side and evacuated by a single pump.

No precise information was given by Van Slyke of the quantity of lime suspension which should be used. It was to be added until the reaction was slightly alkaline. A considerable excess could thus be easily introduced. In previous work it was observed that this excess caused trouble firstly in washing the humin precipitate and secondly in creating violent bumping during oxidation of the humin in the Kjeldahl operation, with loss of many determinations. Excess of lime was also present in the mono-amino acid filtrate and again caused inconvenience in its manipulation.

It was thus desirable to avoid a large excess of lime. By titrating the hydrolysed solution (5 or 10 cc.) using phenolphthalein as indicator, it was possible to calculate the amount needed and to add a slight excess. It was generally found that 0·8 to 1 g. of CaO was thus required and 1 to 1·5 g. was used in the analysis. The lime was finely ground and added in small portions at a time. After adding an equal volume of absolute alcohol, the distillation was started and the volume reduced to 25 to 50 cc.

The humin is then easily and quickly filtered off on a small filter paper of 9 cm. diameter and washed with about 100 cc. of water. If any humin remains adhering to the distilling flask, it is easily dissolved in the sulphuric acid required for the Kjeldahl operation and washed out with water. The water is evaporated off before adding the filter paper with the bulk of the humin precipitate. Frothing and loss of the determination are thus avoided. There is no trouble on account of bumping. In this way evaporation of the filtered solution and washings is avoided before precipitation with phosphotungstic acid.

A short series of experiments was made with baryta water instead of lime. The amide and humin results were the same, but the use of baryta was found disadvantageous in the further stages of the procedure.

Precipitation by Phosphotungstic Acid.

As described by Van Slyke, the filtrate from the humin precipitate is acidified with 18 cc. of conc. hydrochloric acid and precipitated with 15 g. of phosphotungstic acid dissolved in water. The volume is made up to 200 cc. and the flask heated until nearly the whole of the precipitate is dissolved. It is allowed to cool and stand at least two days.

The first possible variation in this procedure is the use of different quantities of phosphotungstic acid. The mono-amino acid filtrate was always found to contain phosphotungstic acid. It is generally stated that the phosphotungstates are soluble in excess of the reagent. Errors may arise on this account, but they are not easily avoided on account of varying quantities of protein that may be used and on account of the different content of hexone bases in the proteins. Only a few experiments were made with this variation. An excess was not found detrimental, but too little gave low results for the hexone bases. 15 g. phosphotungstic acid were therefore added in most experiments. In all experiments the precipitation was made with phospho-24-tungstic acid prepared by us by the method of Wu [1920].

The effects of time of standing of the precipitated phosphotungstates and of the temperature were also investigated (see under Caseinogen II *a* and Gelatin III).

Filtration and Washing of the Phosphotungstates.

In the earliest trial of the method by one of us it was found possible to filter off and wash the precipitate of phosphotungstates on the usual hardened filter paper in a Buchner funnel, and thus avoid the folded paper described by Van Slyke. The filtration is easily effected on a 5 cm. funnel, and if slight suction is maintained during the whole period, the filter paper does not become loosened from the funnel. The filtrate appears clear, but contains very fine particles. A perfectly clear filtrate is obtained by passing the liquid through an ordinary 7 cm. paper.

Owing to the solubility of the phosphotungstates it has seemed to us most probable that the variation in the results would occur for this reason, more especially as the washing and filtration take an appreciable time, from 1 to 3 hours depending on the amount of wash-liquid. The effect of washing the precipitate with 50 cc. and 100 cc. of liquid in 10 cc. portions was first tested. Washing with dilute hydrochloric acid alone was subsequently tried. Slightly lower results for the hexone base fraction were obtained with 100 cc., but the use of hydrochloric acid made no appreciable difference and was adopted for the final set of experiments.

Treatment of the Phosphotungstates.

The procedure adopted was that first described by Van Slyke [1911] in which the precipitate is suspended in water and dissolved in a slight excess of sodium hydroxide. Phosphotungstic acid is removed by adding barium chloride and the filtrate and washings from the barium phosphotungstate evaporated *in vacuo* and made up to 50 cc. Occasionally, it was found that the solution, which gave no further precipitation with barium chloride, on standing over night gave a further reaction. The exact cause of the second precipitation was not discovered, but seemed to depend upon the amount of sodium hydroxide used.

In those experiments in which baryta water was used instead of lime, it was found that the phosphotungstate precipitate contained some insoluble matter, presumably barium phosphotungstate; on this account baryta was not so convenient as lime.

During the course of the later experiments with haemoglobin and caseinogen II the distillate obtained during evaporation was collected in standard acid in the apparatus used for estimating the amide nitrogen. An appreciable and fairly constant amount of ammonia, varying from 0·5 to 1 cc. of $N/10$, was always present. This was most probably derived from the decomposition of the arginine during the evaporation, which occupied several hours.

To avoid this loss of ammonia, the final experiments with caseinogen and gelatin were made on the solution of the phosphotungstates in dilute sodium hydroxide without removal of the phosphotungstic acid and without evaporation. The precipitate dissolved readily on the funnel in from 30 to 40 cc. of N NaOH. The solution and washings were passed through the small 7 cm. filter and made up to 100 cc. The several estimations were equally well made in presence of phosphotungstic acid and bumping did not occur in the Kjeldahl operation if the oxidation was carried out not too rapidly. This procedure has now been adopted by us and has given concordant results with the proteins of eggs [1925, 1].

In whatever way the final solution has been obtained, arginine was estimated by Plimmer's modification [1916] and the amino nitrogen by reaction with nitrous acid for 1 hour [Plimmer, 1924]. Total nitrogen was always estimated in a separate portion.

Treatment of the Mono-amino Acid Filtrate.

In the earliest experiments Van Slyke's description was followed exactly. It was frequently found that during evaporation salts crystallised out and it was not possible to dissolve them in a volume of 200 cc. Their presence caused considerable bumping during the Kjeldahl operation, and errors probably also arose in the making up to volume and measurement of samples.

In order to avoid this trouble, the filtrate was evaporated to dryness *in vacuo* to remove hydrochloric acid as far as possible, and the residue was dissolved in water with the aid of a small quantity of sodium hydroxide, and made up to volume. Later, phosphotungstic acid was removed from the solution by adding barium chloride and treatment according to the process used for the hexone base fraction. Filtration and washing were not so readily effected owing to the gelatinous nature of calcium phosphotungstate. The solution was evaporated *in vacuo* and made up to 100 cc.

During the course of these experiments the distillate, just as in the case of evaporation of the hexone base solution, was collected in standard acid. A fairly constant amount of ammonia, amounting on the average to 0·5 cc. $N/10$ was always found. It is difficult to assign the origin of this ammonia, but it most probably comes from arginine not precipitated by phosphotungstic acid. The presence of arginine in the filtrates was also shown by treatment of a portion with 20 % NaOH. An amount of ammonia, generally 0·8 to 1 cc. $N/10$ for 20 cc. of solution was obtained.

Just as in the case of the hexone base precipitate, the final experiments were made directly. The filtrate and washings were passed through the small 7 cm. filter and made up to 250 cc. The amino nitrogen estimations gave the same results in presence of phosphotungstic acid and hydrochloric acid [see Plimmer, 1925] and bumping only occurred during the Kjeldahl process if the oxidation was done with too large a flame.

This last procedure has again several advantages and has been adopted by us for nitrogen distribution in proteins. The results are very concordant and are rapidly obtained. After standing of the phosphotungstates the analyses are easily completed in two days.

The whole series of results are given in the following tables. In the cases of gelatins I and II, they are calculated to g. of nitrogen per 100 cc., but in the other cases, the actual titration figures and the amount of amino nitrogen in mg. are shown. The differences in the series are thus better seen. The last table gives the mean results calculated in terms of nitrogen per 100 cc.

Gelatin I. T.N. = 0·3598 g. per 100 cc.

Phosphotungstates washed with 50 *cc.*

			Di-amino 50 cc.			Mono-amino 100 cc.	
	Amide N 100 cc. g. N	Humin N 100 cc. g. N	T.N. 5 cc. g. N	Arg. N 20 cc. g. N	Amino N 5 cc. g. N	T.N. 10 cc. g. N	Amino N 5 cc. g. N
	0·0067	0·0007	0·0924	0·0560	0·0465	0·2408	0·1922
	0·0066	0·0004	0·0924	0·0566	0·0465	0·2184	0·1864
	0·0064	0·0007	0·0910	0·0602	0·0461	0·2660	0·2195
	0·0069	0·0007	0·0924	0·0574	0·0455	0·2660	0·2161
Mean	0·0067	0·0006	0·0921	0·0575	0·0462	0·2478	0·2035

Phosphotungstates washed with 100 *cc.*

	0·0063	0·0007	0·0854	0·0560	0·0440	0·2688	0·2626
	0·0069	Lost	0·0868	0·0566	0·0440	0·2660	0·2136
	0·0067	0·0007	0·0812	0·0540	0·0419	0·2716	0·2253
	0·0062	0·0007	0·0854	0·0532	0·0426	0·2716	0·2299
	0·0062	0·0006	0·0840	0·0546	0·0432	0·2744	0·2322
Mean	0·0065	0·0007	0·0846	0·0549	0·0431	0·2705	0·2334

Gelatin II. T.N. = 0·3710 g. per 100 cc.

Phosphotungstates washed with 50 *cc.*

	0·0064	0·0009	0·0910	0·0540	0·0452	0·2744	0·2262
	0·0059	0·0011	0·0896	0·0554	0·0432	0·2744	0·2217
Mean	0·0062	0·0010	0·0903	0·0547	0·0442	0·2744	0·2239

Phosphotungstates washed with 100 *cc.*

	0·0067	0·0004	0·0800	0·0532	0·0429	0·2324	0·2225
	0·0069	0·0006	Lost	Lost	0·0406	0·2324	0·2225
	0·0070	0·0007	0·0882	0·0546	0·0451	0·2688	0·2198
	0·0067	0·0006	0·0896	0·0546	0·0455	0·2716	0·2198
Mean	0·0068	0·0006	0·0856	0·0541	0·0435	0·2513	0·2223

HAEMOGLOBIN. T.N. 10 cc. = 25·35 cc. $N/10$.

Phosphotungstates washed with 50 cc.

			Di-amino 50 cc.			Mono-amino 100 cc.	
	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	T.N. 5 cc. cc. $N/10$	Arg. N 20 cc. cc. $N/10$	Amino N 5 cc. mg. N	T.N. 10 cc. cc. $N/10$	Amino N 5 cc. mg. N
	15·2	9·3	7·5	4·4	6·3851	15·4	9·8302
	15·6	9·3	7·6	4·2	6·4891	15·1	9·9689
	16·2	9·5	7·6	3·8	5·6328	15·5	10·1328
	16·6	9·1	7·1	4·0	6·3093	15·0	9·6518
Mean	15·9	9·3	7·5	4·1	6·2041	15·2	9·8959
Phosphotungstates washed with 100 cc.							
	15·6	9·3	7·4	4·8	5·9942	16·0	10·4542
	15·7	9·4	7·4	4·4	6·4148	15·5	10·0640
	15·9	9·3	6·9	3·6	5·3800	15·9	10·0894
	16·1	9·3	7·2	3·6	5·8605	15·6	9·7421
Mean	15·8	9·3	7·2	4·1	5·9124	15·7	10·0874

CASEINOGEN I. T.N. 10 cc. = 23·65 cc. $N/10$.

Phosphotungstates washed with 50 cc.

			Di-amino 50 cc.			Mono-amino 100 cc.	
	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	T.N. 5 cc. cc. $N/10$	Arg. N 20 cc. cc. $N/10$	Amino N 5 cc. mg. N	T.N. 10 cc. cc. $N/10$	Amino N 5 cc. mg. N
	25·0	2·6	5·6	3·7	4·3975	15·3	8·6643
	24·9	2·5	5·3	3·3	4·2793	15·9	9·1201
	24·9	2·2	5·5	3·7	4·3136	14·8	7·9736
	24·6	2·3	5·6	3·6	4·0707	14·6	7·5927
Mean	24·8	2·4	5·5	3·6	4·2653	15·1	8·3377
Phosphotungstates washed with 100 cc.							
	24·7	2·5	5·3	3·5	4·1391	15·8	9·0533
	24·7	2·6	5·3	3·5	4·2023	15·4	8·7784
	24·8	2·2	5·4	3·3	4·2216	15·1	8·1624
	24·6	2·5	5·4	3·5	4·2962	14·6	8·0517
Mean	24·7	2·4	5·5	3·5	4·2148	15·2	8·5114

CASEINOGEN II. T.N. 10 cc. = 24·0 cc. $N/10$.

(*a*) *Effect of long standing of phosphotungstates. All washed with* 50 *cc.*

			Di-amino 50 cc.			Mono-amino 100 cc.			
	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	T.N. 5 cc. cc. $N/10$	Arg. N 20 cc. cc. $N/10$	Amino N 5 cc. mg. N	T.N. 10 cc. cc. $N/10$	Amino N 5 cc. mg. N	Standing Days	Temp.
	26·1	3·1	5·3	3·4	4·2823	14·4	8·4658	3	0°
	26·2	2·7	5·2	3·6	4·2823	14·8	8·0908	3	room
	26·3	2·6	5·4	3·5	4·5162	14·5	8·4914	15	0°
	26·1	2·5	5·2	3·4	4·3050	14·7	8·6532	15	room
	26·2	2·8	5·5	3·6	4·2190	14·3	8·6195	27	0°
	26·3	2·8	5·4	3·5	4·5382	13·9	8·2193	27	room
	26·1	2·7	5·4	3·5	4·7060	14·2	8·4960	38	0°
	26·3	2·6	5·7	3·4	4·6788	14·3	9·1094	38	room
Mean	26·2	2·7	5·4	3·5	4·4410	14·4	8·5182		

CASEINOGEN II.

(*b*) *Use of* $Ba(OH)_2$ *in place of CaO. All washed with* 50 *cc.*

			Di-amino 50 cc.			Mono-amino 100 cc.			
	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	T.N. 5 cc. cc. $N/10$	Arg. N 20 cc. cc. $N/10$	Amino N 5 cc. mg. N	T.N. 10 cc. cc. $N/10$	Amino N 5 cc. mg. N	Standing Days	Temp.
	25·8	3·0	5·5	3·3	4·7134	14·6	8·9385	3	room
	25·6	2·8	5·2	3·3	4·3759	14·7	9·2850	3	,,
	25·8	2·9	5·0	3·6	5·0122	14·5	9·0594	10	,,
	25·8	3·0	5·4	3·4	4·9907	14·8	9·0197	10	,,
Mean	25·7	2·9	5·3	3·4	4·7730	14·7	9·0756		

CASEINOGEN II.

(*c*) *Use of* $Ba(OH)_2$ *in place of CaO. All washed with* 50 *cc. No evaporation of solutions.*

			Di-amino 100 cc.			Mono-amino 250 cc.	
	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	T.N. 10 cc. cc. $N/10$	Arg. N 40 cc. cc. $N/10$	Amino N 10 cc. mg. N	T.N. 25 cc. cc. $N/10$	Amino N 10 cc. mg. N
	25·9	2·8	5·5	3·6	4·5312	15·0	7·1224
	25·9	2·8	5·3	3·5	4·5895	15·0	7·3439
	25·8	2·5	4·9	3·3	4·1418	14·9	7·2509
	25·7	2·7	4·9	3·6	4·4738	14·9	7·4306
Mean	25·8	2·7	5·2	3·5	4·4341	14·9	7·2869

CASEINOGEN II.

(*d*) *With CaO. No evaporation of solutions. Use of less phosphotungstic acid. Phosphotungstates washed with* 50 *cc.*

							Amount of phospho-tungstic acid. g.
25·8	2·7	4·8	3·6	4·2057	15·3	7·3412	15
25·7	2·7	4·7	3·6	4·3310	15·6	7·5782	15
25·5	3·2	5·0	3·6	4·1887	15·5	7·6412	9
25·7	2·5	4·8	3·9	4·2034	15·4	7·4495	9
25·9	2·6	4·3	3·3	4·5805	15·4	7·3412	8
25·8	2·7	4·3	3·3	4·2636	15·4	7·5347	8
25·9	2·7	4·3	3·3	4·1809	15·7	7·3552	12
26·0	3·0	4·4	3·3	4·2414	15·8	7·7764	13

GELATIN III. T.N. 10 cc. = 29·85 cc. $N/10$.

With CaO. No evaporation of solutions. Different amounts of phosphotungstic acid for precipitation.

Washing of phosphotungstates with $N/10$ *HCl.*

	8·7	0·5	8·5	9·4	5·8345	21·0	9·5880	15
	4·7	0·4	8·3	9·2	5·8902	21·1	9·5947	15
	5·8	0·6	8·9	9·3	6·1891	20·5	9·4574	15
	5·1	0·4	8·7	9·5	6·3654	20·2	9·5297	15
	3·6	0·5	8·4	9·8	5·9600	20·9	9·8637	15
Mean	5·6	0·5	8·6	9·4	6·0478	20·7	9·6067	
	5·6	0·5	8·5	9·8	5·7285	21·0	9·4906	18
	5·7	0·4	8·8	9·5	6·5495	20·2	9·3105	18
Mean	5·6	0·5	8·6	9·6	6·1390	20·6	9·4056	
	6·1	0·5	7·5	9·5	4·8469	21·7	10·2043	12
	7·3	0·5	7·7	9·3	5·3469	21·5	9·8637	12
Mean	6·7	0·5	7·6	9·4	5·0969	21·6	10·0340	

DISCUSSION OF RESULTS.

Amide and humin estimation. Little comment is needed respecting these estimations. The values, except in the one case of gelatin III, are quite concordant and within the margins of experimental error. In this exceptional case, the discrepancies were ultimately found to be due to irregularity in the vacuum during distillation. The use of a slight excess of lime, instead of lime suspension, and the modified receiving apparatus have distinct advantages.

Mean results in terms of g. nitrogen per 100 cc.

			Di-amino						Mono-amino				
	Amide N	Humin N	T.N.	Amino N	Non-amino N	Arginine	Histidine	Lysine	T.N.	Amino N	Non-amino N	Total N sum	Total N found
Gelatin I:													
50 cc. wash	0·0067	0·0006	0·0921	0·0462	0·0459	0·0575	0·0022	0·0324	0·2478	0·2035	0·0443	0·3472	0·3598
100 cc. ,,	0·0065	0·0007	0·0846	0·0431	0·0415	0·0549	0·0005	0·0292	0·2705	0·2334	0·0371	0·3623	,,
Gelatin II:													
50 cc. wash	0·0062	0·0010	0·0903	0·0442	0·0461	0·0547	0·0076	0·0280	0·2744	0·2239	0·0505	0·3719	0·3710
100 cc. ,,	0·0068	0·0006	0·0856	0·0435	0·0421	0·0541	0·0023	0·0292	0·2513	0·2223	0·0290	0·3443	,,
Gelatin III:													
15 g. P.T.A.	0·0078	0·0007	0·1204	0·0605	0·0599	0·0658	0·0158	0·0388	0·2898	0·2402	0·0496	0·4187	0·4179
18 g. ,,	0·0078	0·0007	0·1204	0·0614	0·0590	0·0672	0·0129	0·0403	0·2884	0·2351	0·0533	0·4173	,,
Haemoglobin:													
50 cc. wash	0·0223	0·0130	0·1050	0·0620	0·0430	0·0287	0·0322	0·0441	0·2128	0·1979	0·0149	0·3531	0·3549
100 cc. ,,	0·0221	0·0130	0·1008	0·0591	0·0417	0·0287	0·0303	0·0418	0·2198	0·2017	0·0181	0·3557	,,
Caseinogen I:													
50 cc. wash	0·0347	0·0034	0·0770	0·0426	0·0344	0·0252	0·0232	0·0286	0·2114	0·1667	0·0447	0·3265	0·3311
100 cc. ,,	0·0346	0·0034	0·0770	0·0422	0·0348	0·0295	0·0190	0·0285	0·2128	0·1702	0·0426	0·3278	,,
Caseinogen II:													
Long standing	0·0367	0·0038	0·0756	0·0444	0·0312	0·0245	0·0192	0·0319	0·2016	0·1704	0·0312	0·3177	0·3360
$Ba(OH)_2$	0·0360	0·0040	0·0742	0·0477	0·0265	0·0238	0·0129	0·0375	0·2058	0·1815	0·0243	0·3200	,,
No evaporation	0·0361	0·0038	0·0728	0·0443	0·0285	0·0245	0·0152	0·0331	0·2086	0·1821	0·0265	0·3213	,,

Treatment of phosphotungstates. In the first experiments (gelatin I, II, haemoglobin, caseinogen I) the object was to ascertain the effect of washing the phosphotungstate precipitate with 50 and 100 cc. of wash-liquid. In each case, the amount of nitrogen in the hexone base fraction was slightly less with 100 cc. than with 50 cc., but the difference was insufficient to cause any marked experimental error.

Washing five times with 10 cc. of wash liquid was not only enough, but much more rapid, since the precipitate gradually clogs the filter and delays the process.

In the later experiments washing was effected with dilute hydrochloric acid without phosphotungstic acid. The results were the same. The omission of phosphotungstic acid is an advantage in using our direct procedure of making the determinations without evaporation of the solutions.

No significant difference was noticed by allowing the precipitate to stand at 0° instead of at room temperature. Although the total hexone base nitrogen was greater after standing for 38 days, the long period presents no advantage.

The values for arginine were within the limits of experimental error, and the amino nitrogen figures were consistent. The greatest variations were found in the amino nitrogen figures and these slight differences make the great difference in the final calculations. No appreciable error can be assigned to these estimations which had previously been investigated by Plimmer [1924].

Precipitation with different amounts of phosphotungstic acid. Different amounts of phosphotungstic acid were used in the experiments with caseinogen II and gelatin III. Excess of phosphotungstic acid had no appreciable effect, but too little gave low values for the hexone bases. In those cases in which smaller amounts were used, the filtrate showed the presence of phosphotungstic acid and gave no further precipitation on addition of more reagent. The results indicate that a slight excess of phosphotungstic acid is necessary.

The mono-amino acid filtrate. Errors arising from precipitation and washing of the phosphotungstates accumulate in the mono-amino acid filtrate and augment the differences due to the mono-amino acids alone. The greatest differences in the final calculations are caused by small differences in the amino nitrogen. Compared with the Kjeldahl estimation, the amino nitrogen estimations are too sensitive. It would be advantageous to determine them to the nearest tenth of a mg.

The direct procedure avoiding evaporation of solutions. Loss of ammonia was found to occur during evaporation of solutions. This has been overcome by making the determinations directly. The results were then very consistent and much time was saved. This procedure has been adopted by us as a standard [1925, 2].

Cause of irregularities in the results. Since the individual data are generally within the limits of experimental error, it is very difficult to assign definitely the cause of discrepancies. If the whole procedure be considered, the origin must be located in the precipitation with phosphotungstic acid. Though the conditions are the same, the results vary slightly. Solubility of the phosphotungstates appears to be the chief factor. Corrections for solubility were given by Van Slyke as determined in aqueous solutions of the separate compounds. These values are probably different in the solution of amino acids, and in our calculations have been neglected. Solubility as the chief factor in causing variations is emphasised by observations on keeping the mono-amino filtrate after making the estimations. It was frequently observed that a slight crystalline precipitate slowly formed, and on testing for arginine by boiling with sodium hydroxide a considerable amount of ammonia was evolved [see 1925, 2]. Though incompleteness of precipitation with phosphotungstic acid seems the chief cause of the annoying discrepancies in the final results, the method cannot be condemned on this account. The various data should be taken to the same sub-multiple of the total nitrogen. Estimation of the amino nitrogen to a much higher one, as is usually done, is valueless.

Since the small differences in the actual estimations lead to large differences in the final calculation of percentages, the common practice of returning the data to two places of decimals is entirely misleading. The figures indicate values only within 3 to 5 %.

The values obtained by us for these three proteins on the whole correspond with those found by Van Slyke [1911, 1914], but they show differences amounting to 3 or 4 % noticeable most particularly in the histidine content. No attempt was made in this series of experiments to determine the histidine directly by bromination [Plimmer and Phillips, 1924]. It will be of interest to ascertain how bromination will yield results by the direct procedure.

SUMMARY.

A comparative series of estimations of the nitrogen distribution in the proteins, gelatin, haemoglobin, and caseinogen have been made to ascertain how the discrepancies in results arise during the manipulations of the Van Slyke method. The errors are connected with the phosphotungstic acid precipitation, mainly due to incomplete precipitation of the hexone bases. The greatest differences are found in the amino nitrogen determinations. These are estimated to a higher sub-multiple of the total nitrogen and both should not be recorded beyond tenths of a milligram.

Loss occurs during evaporation of solutions. By adopting a simpler and more rapid procedure avoiding evaporation, concordant results are obtained. These results are within from 3 to 5 % of one another on calculating the nitrogen distribution in percentage of the total nitrogen.

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CXLIV. ANALYSIS OF PROTEINS.

VI. DISTRIBUTION OF NITROGEN IN THE PROTEINS OF EGGS.

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The distribution of nitrogen in the proteins of eggs has not yet been ascertained by the method of Van Slyke. These data are of interest to us in connection with other experiments upon egg proteins and in connection with their value in nutrition. Their determination also gives us a further opportunity of testing our simplified and direct procedure mentioned in the previous paper. A complete series of analyses has been carried out by each of us simultaneously and independently. Concordant results, showing distinct differences in the egg proteins, have been obtained by this procedure.

Experimental.

The proteins used in these experiments were prepared from eggs with white yolks from our hens on a diet of white rice, fish meal, dried yeast and cod-liver oil.

The yolks were separated from the whites and cleaned from adhering white by washing, whilst held in the palm of the hand, under a gentle stream of water. They were then spread upon glass plates and exposed to the air for about 12 hours. The material, thus dried, was scraped off the plates and extracted with cold absolute alcohol, hot absolute alcohol and ether. The fat-free residue, so obtained, formed a slightly reddish-brown powder after grinding in a mortar. This material constituted the egg-yolk protein. No separation into constituent proteins was made for these analyses.

The whites, free from yolk, were broken away from the membranes and poured into $2\frac{1}{2}$ volumes of boiling water containing 0·4 % of acetic acid, and heated for 10 to 15 minutes. The coagulated protein was filtered off, washed with boiling water, placed in two changes of absolute alcohol for dehydration and finally extracted with ether. On grinding in a mortar, a perfectly white powder resulted and formed the egg-white protein.

The acid filtrate from the coagulated white was evaporated to a small volume and poured into two volumes of absolute alcohol. The precipitate of ovomucoid was filtered off, washed with alcohol, redissolved in water and reprecipitated with two volumes of alcohol. It was dehydrated with alcohol and extracted with ether; it formed a white material easily pulverised.

The egg-membrane was prepared from the shells by first treating with very dilute sodium hydroxide to remove adhering white and then with dilute hydrochloric acid to dissolve the calcium carbonate. The residue was freed from acid and treated with absolute alcohol and ether. On drying, the material had an opaque white appearance.

The average yields of these proteins *per egg* and their composition are given below:

	Average yield per egg g.	Moisture %	Ash %	Nitrogen %
Egg-yolk	2·5–3	6·63	5·15	11·43
Egg-white	2·4–2·8	8·68	0·11	12·88
Ovomucoid	0·1–0·3	7·74	1·75	11·23
Egg-membrane	0·2	6·87	0·29	13·16

In each case, 30 g. of protein were hydrolysed by boiling for 36 hours under a reflux condenser with 250 cc. of 25 % hydrochloric acid. The solution was evaporated *in vacuo* to a paste, the paste dissolved in water and the solution again evaporated *in vacuo* to remove hydrochloric acid as completely as possible. The residue was dissolved in water and the volume made up to 1250 cc. The solutions were preserved with thymol.

Total nitrogen was determined in 10 cc. portions and the nitrogen distribution in 100 cc. portions.

The nitrogen distribution was carried out by our simplified and direct procedure without reference to the content of cystine. This procedure may, for convenience, shortly be described.

The 100 cc. portion was measured with a pipette into a double necked distilling flask of 750 to 1000 cc. capacity with side piece bent at right angles. It was made slightly alkaline by the addition, in small quantities at a time, of 1 to 1·5 g. of finely powdered lime, according to its acidity as estimated by titration of a separate 10 cc. portion using phenolphthalein as indicator. After solution of the lime, 100 cc. of absolute alcohol were added and the distilling flask connected to a short double surface condenser set up in a 1500 to 2000 cc. filter flask with Folin tube and bulb attached to the end of the condenser and reaching to the bottom of the filter flask. The filter flask contained excess of $N/10$ acid and sufficient water to cover the bulb amply. Distillation *in vacuo* was carried out at 45 to 50° until the volume in the distilling flask was reduced to 25–50 cc. The excess of acid in the filter flask was then titrated.

The contents of the distilling flask were filtered through a 9 cm. filter paper into a flask with 50 cc. marks up to 200 cc. and the flask and filter washed until the volume reached about 150 cc. Humin adhering to the distilling flask was dissolved in the conc. sulphuric acid used for the Kjeldahl operation and washed out with water. The humin and the filter paper were only added to the sulphuric acid after removal of the water.

The filtrate was acidified with 18 cc. of conc. hydrochloric acid and 15 g. of phosphotungstic acid (prepared by Wu's method), dissolved in a little water, added. The flask was heated until the precipitate had nearly gone into solution, and allowed to cool and stand for 2 to 4 days.

The phosphotungstate precipitate was filtered off on a 5 cm. funnel. For this purpose the newly introduced filters of Jena glass are more advantageous than the Buchner funnel. The precipitate was washed 5 times with 10 cc. of dilute hydrochloric acid (1 : 10), the material being stirred up with the wash liquid and sucked dry each time. The filtrate and washings were passed in turn through a 7 cm. paper and collected in a 250 cc. flask and made up to volume.

The precipitate was dissolved on the filter in 30 to 40 cc. of approx. *N* NaOH, which was first used to dissolve any particles remaining in the flask. This solution was passed through the same 7 cm. filter and collected in a 100 cc. flask. Flask, filter and paper were then washed with changes of water till the volume reached the 100 cc. mark.

The several estimations were then made on the solutions directly. No bumping occurred in the Kjeldahl operation, unless the heating was too rapid. Arginine was estimated by boiling 40 cc. with an equal volume of 40 % sodium hydroxide under a reflux with a Folin tube attached and distilling the ammonia into the bulb after running the water from the condenser. Amino nitrogen was determined in the di-amino fraction by reaction for 1 hour, in the mono-amino fraction for half an hour.

The remains of the solutions from several experiments were mixed together and the above estimations repeated.

Cystine estimations were not made, as the method of estimation is still under investigation.

The results are shown in the following tables. Five separate experiments were made by each of us, but only one series of data is given in order to save space. The two sets were exactly comparable.

These data are followed by calculation of the mean result in terms of nitrogen in g. per 100 cc. and in percentages of total nitrogen.

The tables show the good agreement of the data given by our simplified and direct procedure for a series of analyses on the same solution. The greatest variations are in the estimations of the amino nitrogen; these data should only be taken to tenths of a milligram. In calculating the percentages no allowance has been made for solubilities of the hexone bases.

The individual figures for the several proteins show distinct differences. Egg-white proteins contain more mono-amino nitrogen than the egg-yolk, which has more di-amino nitrogen. It is remarkable that egg-membrane has the highest di-amino figure and has most histidine, and that the histidine is so low in the egg-white and ovomucoid. The lysine nitrogen is very similar in the four proteins.

EGG-YOLK. T.N. = 19·1 cc. $N/10$ per 10 cc.

	Amide N 100 cc. cc. $N/10$	Humin N 100 cc. cc. $N/10$	Di-amino 100 cc. T.N. 10 cc. cc. $N/10$	Di-amino 100 cc. Arg. N 40 cc. cc. $N/10$	Di-amino 100 cc. Amino N 10 cc. mg. N	Mono-amino 250 cc. T.N. 25 cc. cc. $N/10$	Mono-amino 250 cc. Amino N 10 cc. mg. N
	17·2	4·6	5·3	5·6	3·7510	11·8	6·4161
	17·2	4·5	5·1	5·8	3·7971	11·9	6·6222
	17·4	4·4	5·2	5·5	3·6155	12·1	6·4470
	17·4	4·5	5·1	5·7	3·7516	11·8	6·4714
	17·4	4·8	5·1	5·5	3·9707	11·8	6·5124
Mean	17·3	4·6	5·2	5·6	3·7772	11·9	6·4138
Remains of solutions			5·3	5·7	3·8930	12·0	6·4267

EGG-WHITE. T.N. = 21·3 cc. $N/10$ per 10 cc.

	Amide N	Humin N	T.N.	Arg. N	Amino N	T.N.	Amino N
	19·5	4·3	4·5	5·2	3·7508	14·3	7·6055
	19·5	4·1	4·6	5·2	3·9486	14·4	7·6177
	19·3	3·9	4·8	5·0	3·9684	14·3	7·5192
	19·8	4·4	5·0	5·0	3·8355	14·6	8·2888
	19·6	4·2	4·7	4·8	4·0577	14·5	8·3168
Mean	19·5	4·2	4·7	5·0	3·9122	14·4	7·8696
Remains of solutions			4·6	5·2	4·3524	14·5	7·9840

EGG-MEMBRANE. T.N. = 22·5 cc. $N/10$ per 10 cc.

	Amide N	Humin N	T.N.	Arg. N	Amino N	T.N.	Amino N
	15·4	3·5	7·1	7·5	4·7109	14·0	7·0344
	15·4	3·4	7·0	7·8	4·8142	14·0	6·9424
	15·4	3·2	6·9	7·5	4·9015	14·1	6·7937
	15·2	3·4	6·9	7·6	4·6799	14·0	7·0707
	15·2	3·8	6·7	7·6	4·7031	14·0	6·8348
Mean	15·3	3·5	6·9	7·6	4·7619	14·0	6·9352
Remains of solutions			6·7	7·7	5·0575	14·0	7·1443

OVOMUCOID. T.N. = 21·6 cc. $N/10$ per 10 cc.

	Amide N	Humin N	T.N.	Arg. N	Amino N	T.N.	Amino N
	25·8	3·6	4·9	4·5	4·4605	13·9	7·7719
	25·6	3·5	4·7	4·7	4·7943	13·7	7·9421
	25·8	3·7	4·9	4·7	4·6539	13·9	7·9618
	25·8	3·6	5·0	4·6	4·7574	14·0	8·0051
	25·5	3·9	4·8	4·9	4·4818	13·8	8·1423
Mean	25·7	3·7	4·9	4·7	4·6296	13·9	7·9646
Remains of solutions			4·9	4·9	4·5528	13·7	7·9278

MEAN RESULTS IN G. N PER 100 CC.

	Total N	Amide N	Humin N	Di-amino T.N.	Di-amino Arginine N	Di-amino Amino N	Di-amino Non-amino N	Di-amino Histidine N	Di-amino Lysine N	Mono-amino Total N	Mono-amino Amino N	Mono-amino Non-amino N
Egg-yolk	0·2681	0·0242	0·0064	0·0728	0·0392	0·0378	0·0350	0·0084	0·0252	0·1666	0·1624	0·0042
„ white	0·2982	0·0273	0·0059	0·0658	0·0350	0·0391	0·0267	0·0007	0·0301	0·2016	0·1967	0·0049
„ membrane	0·3150	0·0214	0·0049	0·0966	0·0532	0·0476	0·0490	0·0137	0·0297	0·1960	0·1734	0·0226
Ovomucoid	0·3024	0·0360	0·0052	0·0686	0·0330	0·0463	0·0223	−0·0037	0·0356	0·1946	0·1991	−0·0045

MEAN RESULTS IN PERCENTAGES OF TOTAL N.

			Di-amino						Mono-amino			
	Amide N	Humin N	T.N.	Arginine N	Amino N	Non-amino N	Histidine N	Lysine N	T.N.	Amino N	Non-amino N	Total
gg-yolk	9·0	2·4	27·1	14·5	14·1	13·1	3·1	9·4	62·1	60·6	1·6	100·6
gg-white	9·2	2·0	22·1	11·7	13·1	8·9	0·2	10·1	67·6	66·0	1·6	100·9
gg-membrane	6·8	1·6	30·7	16·9	14·8	15·5	4·3	9·4	62·2	55·0	7·2	101·3
vomucoid	11·9	1·7	22·7	10·9	15·3	7·4	−1·2	11·7	64·3	65·5	−1·4	100·6

From these data the content in hexone bases has been further calculated in terms of moisture- and ash-free protein, for comparison with the figures by isolation. In these figures are included data for arginine from the mono-amino fraction (see next paper).

	G. of amino acids per 100 g. of moisture- and ash-free protein				
	Arginine di-amino	Arginine mono-amino	Arginine total	Histidine	Lysine
Egg-yolk	5·7	1·9	7·6	1·4	6·0
Egg-white	5·1	1·8	6·9	0·1	7·4
Egg-membrane	7·4	1·2	8·6	2·2	6·9
Ovomucoid	4·2	1·5	5·7	0	7·6
By isolation [see Plimmer, 1917]:					
Egg-yolk vitellin	—	—	7·5	1·9	4·8
Egg-white crystallised albumin	—	—	4·9	1·7	3·8
Egg-membrane keratin	—	—	—	—	—
Ovomucoid	—	—	—	—	—

The two sets of figures are not in agreement. This is partly accounted for by the different proteins used, though no marked difference has been found between the vitellin and livetin constituting egg-yolk protein, and the crystalline albumin and conalbumin of egg-white. The nitrogen distribution method always gives higher values for lysine. Other data than those given above show 0 or 0·7 % of histidine in egg-white, figures more in agreement with those by nitrogen distribution.

SUMMARY.

The nitrogen distribution in the proteins of eggs has been determined by a shortened and simplified modification of the Van Slyke method. The results are given in a series of tables and comparison is made with the figures obtained by other workers by isolation.

The expenses of these experiments have been covered by a grant from the Government Grant Committee of the Royal Society, to whom we desire to express our thanks.

REFERENCE.

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CXLV. ANALYSIS OF PROTEINS.

VII. DIRECT ESTIMATION OF ARGININE.

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(*Received October 26th, 1925.*)

Arginine is estimated in the Van Slyke method of determining the nitrogen distribution in proteins by decomposition of the di-amino fraction by boiling with sodium hydroxide. As far as is known, no other amino-acid, with the possible exception of cystine, yields ammonia under these conditions. Histidine was found by Plimmer [1916] to give a very small amount. Consequently, it should be possible to determine arginine directly in the solution of hydrolysed protein without precipitation by phosphotungstic acid. On making experiments in this way, it was found that higher results were obtained. On further examination, the mono-amino filtrate from the phosphotungstate precipitate was found to give amounts of ammonia corresponding to the difference. In Part V [1925] it was shown that the errors of the Van Slyke method arose from incomplete precipitation by phosphotungstic acid. If no other amino-acid gives ammonia under these conditions, the arginine content of a protein is the sum of the values obtained from the di-amino and mono-amino fractions, or the value obtained by direct estimation without precipitation by phosphotungstic acid.

EXPERIMENTAL.

The same solutions of hydrolysed proteins were used as described in the previous communications.

Separate portions of 100 cc. were treated with lime to remove amide and humin nitrogen. An equal volume of 40 % sodium hydroxide was added and the solution boiled under a reflux condenser for 6 hours [Plimmer, 1916]. The values for the di-amino fractions were given in the previous papers. The mono-amino fractions were also tested by heating 50 cc. quantities from the experiments in a similar way.

The results, all calculated per 100 cc. of original solution, in terms of cc. $N/10$ were as follows:

	Direct	Di-amino fraction	Mono-amino fraction	Sum of di-amino and mono-amino	N % of total N
Haemoglobin	13·8	10·3	6·0	16·3	13·4
Caseinogen I	12·5	9·0	3·5	12·5	10·4
Caseinogen II	12·0	8·8	3·7	12·5	10·6
Gelatin III	27·8	23·5	3·0	26·5	17·8
Egg-yolk proteins	17·5	14·0	4·5	18·5	19·2
Egg-white proteins	17·4	12·5	4·5	17·0	16·0
Egg-membrane	24·8	19·0	6·0	25·0	22·2
Ovomucoid	21·4	11·8	8·5	20·3	18·6

In all cases, the amount of arginine by direct estimation was higher than by determination in the di-amino fraction. The amount in the mono-amino filtrate in most cases corresponds with the difference. A few values are not concordant. The agreement is better in the experiments in which our direct procedure for the whole series of manipulations was used. The error is greatest in the case of haemoglobin; it was not possible to repeat the experiment as no more of the same solution was available.

The presence of arginine, assuming the absence of an unknown amino-acid, in the mono-amino fraction is further evidence of incomplete precipitation by phosphotungstic acid. This was shown in the previous communication to be a cause of the irregularities in the results by the Van Slyke method. The behaviour of cystine under these conditions has still to be ascertained, but as this compound is not present in gelatin, caseinogen and haemoglobin the only conclusion at present is that arginine as estimated is too low, and that its real value is that obtained by direct estimation or the sum of the figures from the di-amino and mono-amino fractions.

SUMMARY.

Arginine, estimated directly in solutions of hydrolysed proteins by boiling with sodium hydroxide, has a higher value than if estimated in the di-amino fraction precipitated by phosphotungstic acid. On treating the mono-amino fraction in the same way, a value is obtained representing the difference. The arginine value of a protein is the sum of the figures obtained from the di-amino and mono-amino fractions, or the figure obtained directly, assuming that no other amino-acid behaving like arginine is present in proteins.

These experiments have been carried out with the help of a grant from the Government Grant Committee of the Royal Society, to whom we desire to express our thanks.

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CXLVI. TYROSINASE, ITS ACTION ON PHENOLS, TYROSINE AND OTHER AMINO-ACIDS.

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(*Received October 26th, 1925.*)

Happold and Raper [1925] and Raper and Wormall [1925] produced strong evidence that tyrosinase was not capable of deaminising aliphatic amino-acids or tyrosine, and concluded that Bach's [1914] theory of this enzyme action was not correct. Robinson and McCance [1925] working simultaneously over almost the same ground, found that tyrosinase was not capable of oxidising aliphatic amino acids. Happold and Raper moreover and Robinson and McCance both confirmed Chodat and Schweitzer [1913] in this, namely that deamination of the amino-acids followed the addition of certain phenols to the system, and Happold and Raper concluded from their experiments that only those phenols capable of being oxidised to *ortho*-quinones by tyrosinase could act in this way—the amino-acid playing a purely passive part, and undergoing a secondary oxidation by the *ortho*-quinone.

The facts about resorcinol which Robinson and McCance were fortunate enough to detect, and some results reported in this paper show (1) that the system tyrosinase + phenol + amino-acid is a much more complicated one than Happold and Raper supposed, the amino-acid playing an active part in the system, and that probably no *ortho*-quinones are formed; (2) that the action of tyrosinase on tyrosine depends on the fact that tyrosine is both a phenol and an amino-acid; (3) that tyrosinase catalyses the reduction of methylene blue in the presence of an amino-acid + a phenol.

The subject will be dealt with in two parts, firstly, oxidations without enzyme, secondly, the catalysis of these by tyrosinase. All the aerobic experiments were done in Barcroft differential micro-respirometers and the anaerobic experiments in Thunberg vacuum tubes or for temperatures over 40° in test-tubes closed with a rubber bung carrying a glass tube for purposes of evacuation.

I.

Many phenolic substances show this peculiarity. They are readily oxidised by atmospheric oxygen but they are not substances which reduce methylene blue. Batelli and Stern [1921] found this with *p*-phenylenediamine, using thionine, not methylene blue. Their explanation was that the system was a reversible one, and that with only a small amount of thionine present equilibrium was very near the start. Szent-Gyorgyi [1924] explained the same facts on the ground that the atmospheric oxygen was "activated" in Warburg's sense by iron, and on adding tissues a more rapid activation occurred, but that there was no activation of the hydrogen of the *p*-phenylenediamine by the muscle tissue, and therefore methylene blue was unable to act as the hydrogen acceptor even if tissue enzymes were present. Experiments with quinol and catechol have shown that Szent-Gyorgyi's explanation was wrong. Quinol at p_{H} 8 in phosphate or borate buffer was found to take up oxygen at a linear rate which was unaffected by $M/500$ concentration of cyanide (Fig. 1). This excludes activation in Warburg's sense. Further, quinol was found to reduce dinitrobenzene, where activation of the H acceptor by iron is out of the question, but to do so much more slowly than it took up oxygen. The technique used was simply to incubate, after evacuation, a 1 % solution of quinol in buffer solution at p_{H} 8 with a little solid dinitrobenzene. A yellow colour developed over night which turned rose-pink to violet on adding soda. Quinol at p_{H} 8 did reduce methylene blue but only extremely slowly. The reduction was, however, complete and the rate measurable at 80°, so that there are also objections to Batelli and Stern's explanation. The experiments about to be described have made it clear that methylene blue, dinitrobenzene and atmospheric oxygen are not equivalent H acceptors. Wieland [1924] has also had occasion to note a case of this recently. Working on the amino-acid oxidation by charcoal and palladium black Wieland found that methylene blue was not capable of replacing atmospheric oxygen as hydrogen acceptor although dinitrobenzene was capable of doing so. Harger [1924] has confirmed older work that in the formation of amino-quinones from amino-compounds

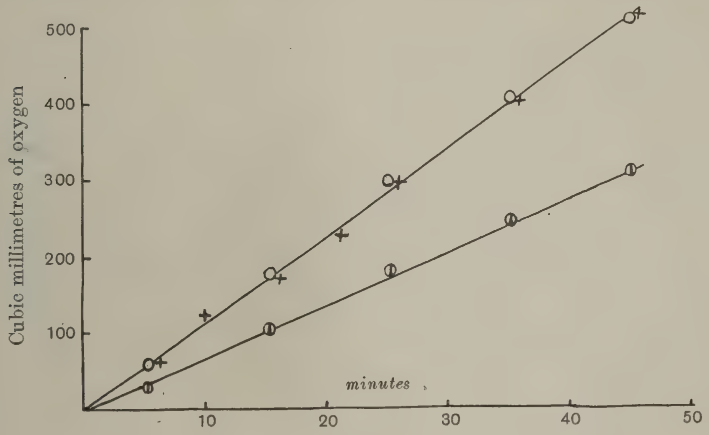

Fig. 1.

○ — ○ 22·5 mg. quinol, 3 cc. borate buffer p_{H} 8.
+ — + 22·5 ,, ,, KCN concentration $M/500$ in 3 cc. borate buffer p_{H} 8.
⦶ — ⦶ 22·5 ,, ,, 22·5 mg. glycine, 3 cc. borate buffer p_{H} 8.

and quinone by oxidation, excess of the latter can act as the oxidising agent and play the part of hydrogen acceptor with aromatic amines and amino-acid esters, but not with aliphatic amines. Atmospheric oxygen on the other hand can act as the hydrogen acceptor in all these cases. In this case quinone and atmospheric oxygen are not equivalent.

In the presence of an amino-acid, quinol and catechol both were found to reduce methylene blue (freely on warming to 80°). An example from some experimental results will make the above statement clear. From solutions of quinol (0·5 % in borate buffer at p_H 8), glycine (1 % in borate buffer at p_H 8) and methylene blue $M/1000$ tubes were filled as follows, evacuated, and placed in a water-bath at 80°.

Table I.

A. 1·5 cc. quinol + 1·5 cc. buffer + 0·3 cc. meth. blue: slightly reduced in 2½ hours
B. 1·5 cc. quinol + 1·5 cc. glycine + 0·3 cc. meth. blue: colourless in 21 mins.
C. 3 cc. buffer + 0·3 cc. meth. blue: no change in 2½ hours

This action of amino-acids was found to be specific; aliphatic amines (methylamine and ethylamine), aniline, urea and ammonia were unable to act in this way, when the p_H was carefully adjusted. Glycine anhydride had a very small effect, due no doubt to hydrolysis. This reaction has been carried out using larger quantities of the reagents to determine if the amino-acids were oxidised during the reduction of the methylene blue. 10 mg. catechol, 10 mg. glycine, 1 cc. phosphate buffer and 4 cc. $M/50$ methylene blue in phosphate buffer (p_H 8) were heated together in a vacuum, one control tube being similar but containing no methylene blue and another containing no catechol. After complete reduction of the methylene blue the amino-N content of all the tubes was estimated in the smallest form of the Van Slyke apparatus and found to be exactly the same in all. In a second experiment ammonia was estimated by Nessler's method, and no appreciable increase was detected in the experimental tube. In a third experiment catechol was replaced by quinol with similar results. It is almost certain that the oxidation quinol → quinone is not what is catalysed by the amino-acid, for if it had been the amino-acid would have been found to have been oxidised [Traube, 1911]. *p*-Cresol alone was found practically not to reduce methylene blue at all, but to do so slowly in the presence of glycine. These reactions were found to be quite unaffected by $M/500$ KCN. Tyrosine being both an amino-acid and a phenol was found to reduce methylene blue slowly. The experimental evidence is summarised in Table II. The tyrosine was recrystallised repeatedly and the rate of reduction was found to be unaffected by this treatment. 0·5 g. tyrosine was dissolved in 20 cc. phosphate buffer at p_H 8 by warming. 3 cc. (in duplicate) were added to 0·3 cc. $M/1000$ methylene blue in a tube and evacuated, placed in a water-bath at 80° and the reduction time noted. Equimolecular solutions of the other substances tested were treated in duplicate in an exactly similar way.

Table II.

Substance	Reduction time
Alanine	No reduction
Leucine	,,
Tryptophan	Over 5 hours
Glycine	No reduction
Phenylalanine	,,
p-Cresol	,,
Tyrosine	1 hour 20 mins.

Aerobically quinol was found to have a rapid oxygen uptake alone as already stated. Addition of an amino-acid, however, did not accelerate the oxygen uptake but retarded it, and ammonia and aliphatic amines had the same effect. Both these observed results were the reverse of those obtained with methylene blue, and already described. Both seem to have their origin in the fact that there is something specific about each of these hydrogen acceptors. Fig. 1 shows the results of one out of six experiments which all gave identical results. It should be contrasted with Table I. Aerobic oxidation of catechol was found to be slower than that of quinol and the presence of ammonia to give a similar but less marked retardation of the oxygen uptake. The addition of glycine gave at first a slower oxygen uptake, as with quinol, but this oxygen uptake became faster in an autocatalytic manner. Probably further secondary oxidations follow in this case which do not occur with quinol, and the initial effect of the glycine is exactly the same in both cases. $M/500$ KCN does not affect any of these oxidations (Fig 1).

Two reactions must be capable of taking place simultaneously in the system phenol + amino-compound + hydrogen acceptor: (1) the same oxidation of the phenol as takes place in the absence of the amino-compound: (2) the oxidation of the coupled system. This alone is appreciable with methylene blue in the case of quinol, and there is a further specificity in that only amino-acids seem capable of inducing it with this hydrogen acceptor (only α-amino-acids have been tried). Aerobically both (1) and (2) take place, and amines and amino-acids exert apparently the same action. There is no unknown factor such as an enzyme present, so that all the results must be due to differences in the hydrogen acceptors. The results indicate that the various substances used as hydrogen acceptors may have certain properties in common, but that each possesses a very marked specificity of its own which is so far quite unexplained, but which must depend ultimately upon chemical structure. So much so is this the case that sometimes this chemical structure seems more important than oxidation potential. This is particularly true of the case reported and confirmed by Harger [1924] where atmospheric oxygen oxidises both aromatic and aliphatic amines with quinone to form amino-quinones, and the excess of quinone (which has an oxidation potential) will effect the former but not the latter.

The colour formed by these phenol oxidations is so striking and has formed such a large part of the evidence from which conclusions have been drawn in the past, that the following observations are important, especially if compared with the observed rate of oxygen uptake. On adding a solution of glycine to one of catechol (both at p_H 8) there was an instantaneous violet coloration. This was due to impurities (iron, etc.) in the glycine, took place in a vacuum and, as the Barcroft results showed, had no connection with the oxygen uptake. In time the solution of catechol + glycine became darker than the solution of catechol alone, and finally went a reddish brown while the control was still practically colourless. When two bottles, one containing quinol and the other quinol + glycine, were shaken simultaneously the one containing glycine quickly became pink, then red, and later red brown. The cause of this was almost certainly the formation of amino-quinones which are red compounds thus: quinol → quinone + 2 amino-acid → amino-acid quinone.

II.

Two sources of tyrosinase have been used: (*a*) the same enzyme and method of preparation used in a previous paper [Robinson and McCance, 1925], (*b*) tyrosinase from the meal-worm. This was freshly prepared for each experiment, as the preparation soon lost its activity on keeping. The worms were ground up very fine in a mortar with a little water, the solid material washed six times with water, and separated from the washings each time by the use of a centrifuge. The washings were disregarded and the fine suspension used as the enzyme solution. The action of each enzyme appeared to be the same. The suspension of meal-worm used as a source of tyrosinase contained a powerful peroxidase.

The reduction of methylene blue in each of the following systems was found to be catalysed by tyrosinase: quinol + glycine, catechol + glycine, and especially *p*-cresol + glycine (all in buffer at p_H 8). Without enzyme quinol (or catechol) + glycine reduced methylene blue much faster than *p*-cresol + glycine but in the presence of the enzyme these relative rates were reversed and the same amount of methylene blue was reduced very much faster by *p*-cresol + glycine. *p*-Cresol is of course much more akin structurally to tyrosine than is quinol. *o*-Cresol + glycine practically did not reduce any methylene blue with or without enzyme, and *m*-cresol + glycine reduced methylene blue almost as fast as *p*-cresol in the presence of the enzyme. Tyrosinase can act therefore by accelerating the anaerobic oxidation of a phenol in the presence of an amino-acid and it is reasonable to suppose from what is known of other enzymes that this is the method by which it acts aerobically. In other words tyrosinase exerts its catalytic action by hydrogen activation. At the same time tyrosine + tyrosinase did not reduce methylene blue any faster than tyrosine alone. These experiments have been done most carefully: some tubes were kept 8 days in a high vacuum with only small amounts of methylene blue present, and Raper and Wormall's [1923] results confirmed. There can, however, be little doubt that in the presence of a suitable hydrogen acceptor tyrosinase would act on tyrosine anaerobically.

Aerobic experiments with tyrosinase afforded further evidence that the amino-acid plays an active part in the system. The oxidation of *p*-cresol is enormously accelerated by tyrosinase (its rate of oxidation without any enzyme is negligible). The rate of oxygen uptake was, however, still further increased by the addition of glycine.

Nature of the effects of amino-compounds on phenol oxidation.

Quinol has been found to form various addition compounds with aliphatic amines [Harger, 1924] and it is probable that compounds of a similar nature are formed by amino-acids and ammonia but they have not yet been isolated. It is possible that these compounds form the first step in the formation of amino-quinones. Such an addition compound might be more easily attacked by oxidising agents than the simple quinol, and in fact have quite different properties. No other phenols have so far been reported to form these addition compounds, but catechol and resorcinol have now been found to form well defined crystalline compounds of this type (see experimental section at the end of this paper), and when solutions of *p*-cresol and various amines were mixed there was an evolution of heat but no crystals were obtained on evaporation. The same was true of leucine ethyl ester with all the phenols tried, namely quinol, *p*-cresol, *m*-cresol and catechol. With Mr Dixon's assistance the effect on the reduction potential of adding glycine to a solution of catechol or quinol was tested and found to be nil. This was unexpected in view of the marked effect the same addition would have had on the reduction of methylene blue and the rate of oxygen uptake, besides the chemical evidence of a phenol-amine addition compound. The exact nature of the effect exerted by an amino-compound on the oxidation of a phenol, and the chemistry of that oxidation must remain undecided for the present.

The effect of cyanide and pyrophosphates.

Only the meal-worm tyrosinase has been used for these experiments. Both KCN and pyrophosphates have no effect on the enzyme-free oxidations already described. Pyrophosphates ($M/500$) were found to inhibit slightly both the methylene blue reduction and oxygen uptake of *p*-cresol + glycine + tyrosinase. KCN is extremely toxic to the enzyme, and exerts this effect both with methylene blue and oxygen. The following is a typical experiment. Four vacuum tubes were made up as follows: *A* and *B*; 10 mg. *p*-cresol, 10 mg. glycine, 1·5 cc. borate buffer p_H 8, 0·3 cc. methylene blue $M/1000$, 1 cc. enzyme, 0·5 cc. H_2O: *C* and *D*; the same with the addition of KCN (titrated to p_H 8) instead of water to make a final concentration of $M/500$. After evacuating, all four were placed in a water bath at 38°. *A* and *B* were completely decolorised in $1\frac{3}{4}$ and 2 hours respectively, *C* and *D* were still blue after 5 hours. There was, however, aerobically a very unusual feature of this inhibition by KCN, for its effect wore off after a time and the system began to take up oxygen, and this moreover very much sooner and faster when glycine was present. This was definitely not due to hydrolysis of the KCN as time went on, and in fact the addition of more KCN when the latent period was over seemed to make very little difference. The enzyme seemed almost to have become immune to KCN (Fig. 2). This inhibition is obviously a very complicated mechanism and would have provided material for a complete investigation. One difficulty would have been to standardise the conditions, for the enzyme preparation seemed to lose strength on keeping, and the length of the latent period was found to depend among other things on the relative proportions of the KCN and enzyme. Some of the results obtained are here given briefly: (*a*) the KCN inhibition of the oxygen uptake of *p*-cresol + tyrosinase was greatly shortened by the addition of glycine to the system; (*b*) the rate of oxygen uptake and the amount of oxygen taken up after the latent period depended on the amount of glycine present; (*c*) the red colour formed by this oxidation in the solution and, therefore, probably the products of the oxidation, appeared identical with those formed in the absence of KCN.

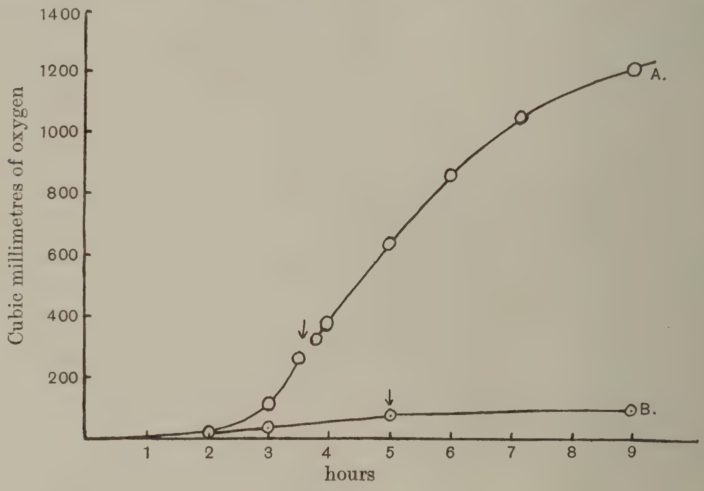

Fig. 2.

A. 10 mg. *p*-cresol. 10 mg. glycine. 1·5 cc. borate buffer p_H 8. KCN (total concentration $M/500$). 0·5 cc. H_2O. 1 cc. tyrosinase suspension.

B. 10 mg. *p*-cresol. 1·5 cc. borate buffer p_H 8. (KCN concentration $M/500$.) 0·5 cc. H_2O. 1 cc. tyrosinase suspension.

At ↓ concentration of KCN was increased to $M/250$.

Perhaps the most interesting points at present are, (*a*) that there is a marked inhibition by KCN which is not an effect of iron, (*b*) that this latent period and curve of oxygen uptake is exactly the same as that obtained with resorcinol and glycine, resorcinol [Gortner, 1911] being known to exert an inhibitory effect on tyrosinase. It almost seems as if the amino-acid exerted a double function, (*a*) an effect on the phenol, (*b*) a protective effect on the enzyme.

The nature of tyrosinase.

Onslow and Robinson [1925] have advanced a theory of the action of tyrosinase, which is an extension of Onslow's "catechol" theory of oxidations. Essentially the theory is that either all samples of tyrosine contain traces of an *ortho*-dihydroxy-compound as an impurity, or that the enzyme contains traces of active oxygen. If this were true, the initial rate of oxygen uptake would depend entirely on the amount of this impurity and the curve of oxygen uptake would be autocatalytic, which has never been proved. Moreover, the following experimental evidence makes it practically certain that the oxidation of *p*-cresol, tyrosine, resorcinol, quinol, etc., with or without enzyme, and with or without glycine, or other amino-compound, does not lead to the formation of *o*-dihydroxy substances. Catechol and all *o*-dihydroxy substances combine quantitatively with boric acid to give compounds with strongly acid properties [Lambert, 1889]. This property of borates is so definite that it has formed the basis of two methods for the volumetric estimation of boric acid. All the above phenols undergoing oxidation spontaneously or under the influence of tyrosinase have been found to give quantitatively similar oxygen uptakes in borate or phosphate buffer at p_H 8 and the p_H after the oxidation is the same in both cases, *e.g.* in both 1 mol. *p*-cresol takes up 3 atoms of oxygen. On the contrary, catechol, 0·5 % in borate buffer p_H 8, gives a strongly acid solution. These same facts also disprove Happold and Raper's [1925] theory that deamination of the amino-acid follows only if an *ortho*-quinone is formed in the oxidation of the phenol.

Raper and Wormall [1925] found that tyrosinase would not act upon *p*-hydroxyphenylpyruvic acid + NH_3, though of course it would act readily on *p*-hydroxyphenylalanine (tyrosine). Methylene blue has been found to be reduced by a phenol + an amino-acid but not by a phenol + NH_3, and this reduction is catalysed by tyrosinase. It would seem that in both these cases the presence of the amino-acid was necessary to effect the oxidation of the phenol. In tyrosine both the oxidisable phenol group and the "co-enzyme" amino-group are combined in the same molecule. This seems to be the reaction catalysed by tyrosinase, though the chemistry of this oxidation remains to be determined.

Robinson and McCance [1925] reported that in the systems catechol (or *p*-cresol) + amino-acid + tyrosinase, the oxygen used for the amino-acid agreed quantitatively with the reduction of the amino-N but much less ammonia than this was set free. The explanation of this is possibly as follows. Some of the amino-acid is involved in the formation of the phenol-amino-compound (such as an amino-quinone); this would lead to a decrease in the free amino-N of the system but not to any liberation of free ammonia. Hydrogen peroxide probably is simultaneously formed and this would react with the peroxidase and more amino-acid to give both a reduction of amino-N and an increase of free ammonia. The discrepancy does not seem to be due to the formation of cyanates, for none were detected by the methods described by Fearon and Montgomery [1924] in a solution of *p*-cresol, glycine and tyrosinase which had been aerated 2½ days at p_H 8.

EXPERIMENTAL SECTION.

Preparation of the addition compound (catechol)$_2$-diethylamine.

2·5 g. catechol were dissolved in 50 cc. absolute ether and 5 cc. diethylamine added. Crystals very soon began to separate. These were filtered off, washed twice with small quantities of absolute ether and recrystallised from warm benzene. The crystals were white rectangular plates, very soluble in water and ether, less soluble in benzene and light petroleum. M.P. 78–79°.

Analysis. Found: C, 66·03; H, 7·93; N, (1) 4·6, (2) 4·8 %.

Calc. for (catechol)$_2$-diethylamine: C, 65·53; H, 7·86; N, 4·78 %.

After keeping a month the crystals had turned brown. The compound was completely dissociated in acid solution and the amine could be titrated.

Preparation of the addition compound (resorcinol)$_2$-diethylamine.

1 g. resorcinol was dissolved in absolute ether and diethylamine added drop by drop until the granular precipitate which appeared momentarily with each drop persisted. On standing a heavy precipitate appeared. This proved to be readily soluble in excess of diethylamine. The precipitate was filtered off, washed and recrystallised from benzene. The crystals obtained were white needles with similar solubility to the catechol compound. M.P. 115°.

Analysis. Found: C, 65·55; H, 7·71; N, 4·75 %.

Calc. for (resorcinol)$_2$-diethylamine: C, 65·53; H, 7·86; N, 4·78 %.

The crystals did not turn brown on keeping, even after 6 months.

In an endeavour to discover the nature of the compound formed by the action of tyrosinase on *p*-cresol alone, the addition compound of quinone-(*p*-cresol)$_2$ analogous to the known pheno-quinone compound was synthesised. This compound has an orange colour identical in appearance with that formed by the *p*-cresol tyrosinase system, but the properties of the two solutions are not the same.

1·5 g. quinone dissolved in hot light petroleum were added to 6 g. of *p*-cresol also dissolved in hot light petroleum. On cooling a heavy crop of orange-red crystals was obtained and recrystallised from the same solvent. M.P. 63·5°. The crystals were needles and always had a faint smell of quinone.

Analysis. Found: C, 73·78; H, 6·34 %.

Calc. for quinone-(*p*-cresol)$_2$: C, 74·1; H, 6·18 %.

SUMMARY.

1. Experiments have shown that there may be a marked specificity among the various substances classed as H acceptors. Their chemical structure seems to be the basis of this and any further explanation seems impossible at present.

2. Tyrosinase catalyses the reduction of methylene blue by a solution at p_H 8 of p-cresol + glycine. Tyrosinase, therefore, acts by hydrogen activation.

3. The action of tyrosinase on tyrosine depends upon the fact that tyrosine is both an amino-acid and a phenol, and though the amino-acid does not undergo any deamination, it appears to act as a co-enzyme for the oxidation of the phenol. Similarly, in the system p-cresol + amino-acid + tyrosinase, the amino-acid does not merely undergo secondary oxidation but plays an active part in the system.

4. The spontaneous oxidation of all the phenols examined (alone or together with amino-compounds) has been shown to be independent of KCN. $M/500$ KCN strongly inhibits both the aerobic and anaerobic action of tyrosinase.

5. Addition compounds of catechol and resorcinol with diethylamine, and a p-creso-quinone compound analogous to the known pheno-quinone, have been prepared for the first time.

The author takes this opportunity of thanking Sir F. G. Hopkins for his help, criticism and encouragement.

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CXLVII. THE CHEMICAL CONSTITUTION OF SPERMINE.

II. THE METHYLATION OF SPERMINE.

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(*Received October 27th, 1925.*)

In a previous communication [1924] it was shown that the base spermine can be isolated from a variety of tissues and has the formula $C_{10}H_{26}N_4$.

Of the various methods of attacking the problem of its chemical constitution which have been tried, the most promising appears to lie in the exhaustive methylation of the base. It has been found that by this procedure ten methyl groups are taken up by spermine, giving a quaternary ammonium base $C_{20}H_{50}N_4(OH)_4$ and indicating that two of its nitrogen atoms are primary whilst the other two are secondary. The degradation of this base yields at least two simpler bases, the identification of which will be attempted with the larger amounts of spermine now available.

EXPERIMENTAL.

Methylation of spermine. 1·59 g. spermine hydrochloride were dissolved in 5 cc. water; 40 cc. 2 % KOH in methyl alcohol and 5 cc. CH_3I were then added. The mixture was heated to 40° in a water-bath under a reflux condenser until its reaction was neutral to litmus, which occurred in 30–45 minutes. 10 cc. 4 % KOH in methyl alcohol were then added and the heating continued. At about half-hourly intervals, the solution having become neutral again, similar quantities of KOH were added; in all, fourteen such additions and three of 4 cc. CH_3I were made. To the practically neutral solution water was added to dissolve the crystalline material which had separated and the methyl alcohol and excess of methyl iodide were distilled off *in vacuo*. The iodides in the aqueous residue were converted into chlorides by shaking with excess of freshly precipitated silver chloride, and to the filtrate (300 cc.) from this was added a saturated aqueous solution of mercuric chloride. This precipitates the very sparingly soluble mercurichloride of decamethylspermine. After standing the mercurichloride was filtered off, washed with water and dried in a vacuum desiccator. Yield 7·05 g. This salt was suspended in 500 cc. water, heated on the water-bath and the mercury was removed by H_2S. The HgS was suspended in 500 cc. hot water and again treated with

H_2S to ensure complete decomposition. The combined filtrates were concentrated on the water-bath to about 150 cc. when excess of sodium picrate solution was added. The picrate, after standing, was filtered off, washed with a little water and dried at 90°. Yield 5·7 g. This is a practically theoretically quantitative yield. After recrystallisation from water 5 g. of the pure picrate were obtained from which a solution of the chloride was made in the usual way and the following salts prepared.

Decamethylspermine chloroaurate: $C_{20}H_{50}N_4\ (AuCl_4)_4$. This salt is very sparingly soluble in water, but is readily soluble in mixtures of water and acetone from which it crystallises in glistening, diamond-shaped plates. Recrystallised from 15 % HCl it is obtained as short, prismatic needles. M.P. 278–280° with decomposition. It contains no water of crystallisation.

Analysis. 0·1300 g. gave 0·0598 g. Au.

Au found, 46·0 %; calc. 46·30 %.

Decamethylspermine chloroplatinate: $C_{20}H_{50}N_4\ (PtCl_6)_2$. This salt is very insoluble in water and alcohol. Recrystallised from 15 % HCl it is obtained as a dull orange powder, the crystalline form of which resembles that of ammonium chloride. M.P. 286–288° with decomposition. It contains no water of crystallisation.

Analysis. 0·1757 g. gave 0·0588 g. Pt.

Pt found, 33·47 %; calc. 33·56 %.

Decamethylspermine tetrapicrate: $C_{20}H_{50}N_4\ (C_6H_2O_7N_3)_4$. This salt is very sparingly soluble in water (about 1 part in 6500 at room temperature), from which it crystallises in long, thin, flat needles. M.P. 272–274° with decomposition.

Analysis. 0·08135 g. gave 0·1399 g. nitron picrate.

Picric acid found, 72·80 %; calc. 72·82 %.

Decamethylspermine mercurichloride. This salt is very insoluble in cold water and can be recrystallised from dilute HCl in star-shaped groups of thick prisms. M.P. 268°.

The *chloride* of the ammonium base is very hygroscopic; with a solution of iodine in potassium iodide it gives a chocolate-brown crystalline precipitate, and a crystalline precipitate with an alcoholic solution of zinc chloride.

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CXLVIII. NOTES ON SPERMINE.

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I. THE IDENTITY OF MUSCULAMINE, NEURIDINE AND GERONTINE WITH SPERMINE.

(*a*) *Musculamine.* Étard and Vila [1902] isolated this base as a benzoyl compound from hydrolysed calf's muscle, and gave it the formula $C_8H_{21}N_3$. Posternack [1902] pointed out the agreement of the analytical figures with $C_5H_{14}N_2$ and suggested the identity of musculamine with cadaverine, a suggestion which was afterwards adopted by Étard and Vila [1903]. We have obtained the same benzoyl compound from a dilute acetic acid extract of calf's muscle, but were unable to isolate it from the hydrolytic products of the extracted muscle. The substance is therefore not a cleavage product of muscle protein as previously assumed. Moreover we were able to identify the base as spermine by means of its characteristic insoluble phosphate, the picrate (M.P. 250°) and the chloroplatinate (M.P. 245°). The analytical figures given by Étard and Vila agree with the formula of spermine, $C_{10}H_{26}N_4$, as established by Dudley, M. C. Rosenheim and O. Rosenheim [1924].

(*b*) *Neuridine.* A base of the composition $C_5H_{14}N_2$ was obtained by Brieger [1885] from fresh human brain and named neuridine on account of its assumed relation to neurine. Its alleged occurrence amongst the products of putrefaction was no longer maintained by Brieger himself after his subsequent discovery of cadaverine (pentamethylenediamine), which has the same elementary composition. We prepared the base from fresh ox brain and found it to be identical with spermine (insoluble phosphate of characteristic crystalline form, chloroaurate, M.P. 225°). We are able to confirm Brieger's description of the properties of the picrate and its behaviour on heating, which furnish an additional proof of the identity of neuridine and spermine.

(*c*) *Gerontine.* In histological sections of dog's liver, Grandis [1889] found transparent, fairly insoluble crystals, which he isolated by mainly mechanical methods [1890] and identified as the phosphate of a base having the composition $C_5H_{14}N_2$. The chloroplatinate was analysed and a description of the picrate is given, unfortunately without its melting point. According to Grandis the base is not identical with pentamethylenediamine. Although we have not repeated the tedious mechanical isolation of the crystals, as described by

Grandis, we had no difficulty in obtaining similar crystals from dog's liver by the chemical methods previously described for the isolation of spermine phosphate [Dudley, M. C. Rosenheim and O. Rosenheim, 1924]. In spite of slight discrepancies in the described properties of gerontine and spermine, which may easily be due to the inherent difficulties of Grandis' method of isolation, we feel justified in considering the two bases as identical.

II. The distribution of spermine.

In our attempts to find a suitable material for the preparation of spermine in reasonable quantity, we carried out a number of experiments which furnish a fairly reliable picture of the distribution of this interesting base. Usually 3 to 5 kg. of material were worked up by one of the methods previously described [Dudley, M. C. Rosenheim and O. Rosenheim, 1924]. The figures given in the following table represent the actual yield of once recrystallised spermine phosphate, expressed in mg. per 100 g. of fresh material and arranged in the order of yield.

Semen (man)	260 mg. %	Testis (bull)	6 mg. %
,, (bull)	0	Thymus (calf)	5 ,,
Pancreas (ox)	25–30 ,,	Adrenal (ox)	5 ,,
Liver ,,	16 ,,	Thyroid ,,	3 ,,
Kidney ,,	15 ,,	Blood (ox) whole	0
Ovary (cow)	14 ,,	,, defibrinated	0
Muscle (calf)	12 ,,	,, serum	0
,, (sheep)	4 ,,		
,, (ox)	2 ,,	Milk (cow)	0
,, (horse)	2 ,,	Egg (hen)	0
Spleen (ox)	11 ,,		
Lung ,,	7 ,,	Yeast (Lebedeff)	10 ,,
Brain ,,	7 ,,	,, (distillers)	7 ,,
		,, (marmite)*	40 ,,
		,, (baker's)	0

* The spermine content of "marmite" is very variable. The figure represents the maximum yield, whilst some batches contain only traces.

The absence of spermine from bull's semen is striking. We were unable to isolate it from 30 cc. of the material by the same method which yields weighable amounts when carried out with only 5 cc. of human semen [Rosenheim, 1924]. This result confirms the accuracy of the micro-chemical picric acid test (Barberio), which is negative with animal semen [Baecchi, 1912].

Apparently calf's muscle is richer in spermine than that of the fully grown animal. In the case of a six weeks old pig, however, we found no greater percentage of spermine in its tissues than occurred in those of an adult animal. The fact that the existence of spermine in muscle has been overlooked by Kutscher and his school is probably explained by their use of tannin for the preliminary purification of meat extracts.

III. The significance of spermine.

No systematic study of the physiological properties of spermine has yet been made, but such information as we have at present throws no light on the rôle of spermine in the organism. Its absence from bull's semen appears to indicate that it is not essential for fertilisation in general, whilst the fact that it is not present either in the egg or in milk implies that it is not essential to the development and growth of the young animal. This view is supported by the result of an experiment carried out by Prof. J. C. Drummond, who found that spermine cannot replace vitamin B (yeast). Prof. R. A. Peters has performed some tests which prove that spermine, which is present in yeast, has no anti-neuritic activity, whilst Prof. A. Harden has informed us that experiments made to ascertain whether spermine phosphate could function as a co-enzyme in alcoholic fermentation yielded negative results.

Our best thanks are offered to these gentlemen for their kindness and interest in making the experiments and for their permission to quote the results.

We are also indebted to Dr J. Golding of the National Institute for Research in Dairying, Reading, for supplying us with a quantity of bull's semen.

SUMMARY.

1. Spermine is shown to be identical with musculamine, neuridine and gerontine, bases which have been obtained from calf's muscle, human brain and dog's liver, respectively, by earlier investigators.

2. The yields of spermine obtained from various animal tissues and yeast are tabulated. It is shown to be absent from bull's semen, ox blood, cow's milk and hen's egg.

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CXLIX. A FURTHER NOTE ON THE KINETICS OF ENZYME ACTION.

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(*Received October 29th, 1925.*)

The equation of Michaelis and Menten [1913], to which reference was made in a previous note [Briggs and Haldane, 1925], holds for the case where one molecule of enzyme combines with one molecule of substrate, as the first mentioned authors assume. It holds also for other cases.

Euler and his co-workers [1923, 1924] have obtained evidence that the silver equivalent of invertase is about one-fourth of the molecular weight of the enzyme. The same holds for the bromine and sulphur equivalents. If the saccharose equivalent also is less than the molecular weight, the equation of Michaelis and Menten no longer holds unless we elaborate the picture.

Assuming the sugar equivalent to be one-fourth of the molecular weight, that is, each enzyme molecule is capable of accommodating four sugar molecules, there will be five species of enzyme molecules in a solution containing invertase and sugar: enzyme molecules free from sugar, molecules with one molecule of sugar attached, and so on up to those with four molecules attached. The fraction of the total enzyme molecules in these five states being x, y, z, u and v, the fraction of the total enzyme which is active (e_s) is equal to $\frac{y+2z+3u+4v}{4}$; a molecule of enzyme with two sugar molecules attached being twice as effective as a molecule with one, and so on. If we assume that only one molecule of sugar can be gained or lost at a time, then it can readily be shown that the expression for the fraction of enzyme active is

$$4e_s = \frac{sk_s^3 + s^2k_s^2 + s^3k_s/2 + s^4/6}{k_s^4 + sk_s^3 + s^2k_s^2/2 + s^3k_s/6 + s^4/24};$$

s is the concentration of substrate and $k_s = \frac{k_2 + k_3}{k_1}$*. Values of fractional activity are given in the table, column A. These depart considerably from the values calculated from the equation $e_s = s/(s + k_s)$, the equation of Michaelis and Menten.

		e_s	
s/k_s	A	B	C
$\frac{1}{4}$	·057	·062	·068
$\frac{1}{2}$	·125	·121	·130
1	·246	·225	·230
2	·453	·373	·371
4	·690	·540	·539
8	·854	·683	·693
16	·933	·813	·812

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* k_2 is the velocity constant of detachment of sugar molecules, k_3 that of conversion of combined molecules into invert sugar.

If we amplify our picture by supposing that sugar molecules can be combined to enzyme molecules so that the sugar is linked at two points as well as at one, as we assumed above, the number of species of enzyme molecules is increased to nine. Assuming that only those sugar molecules attached at one point can break down into invert sugar, the fractional activity of the enzyme is given by the expression

$$4e_s = \frac{sk_i^2k_s^3 + s^2k_i^2k_s^2 + s^3k_i^2k_s/2 + s^4k_i^2/6 + s^2k_ik_s^3 + s^3k_ik_s^2}{k_i^2k_s^4 + sk_i^2k_s^3 + s^2k_i^2k_s^2/2 + s^3k_i^2k_s/6 + s^4k_i^2/24 + sk_ik_s^4 + s^2k_ik_s^3 + s^2k_s^4/2 + s^3k_ik_s^2/2},$$

where k_i is the ratio of the velocity constants of attachment and detachment of the second class of sugar molecules. The fractional activity when $k_i = 2k_s$ is shown in column B. In column C are given the values assuming the equation of Michaelis and Menten to hold, the value of k_s being 3·25 times that used in our equation. It will be seen that the fit is quite good; it is comparable with the fit obtained by Kuhn [1923] for the Michaelis equation and fractional activity of invertase.

In the derivation of these equations the assumption was made that the probability of the combination of a substrate molecule with an enzyme particle is independent of the number of vacant places on the latter. Should the probability be proportional to this number then the first equation becomes identical with the Michaelis equation.

This subject is being treated in greater detail by the author in an account of enzyme action which it is hoped will appear shortly.

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CL. A NOTE ON THE LIPOCHROMES PRESENT IN CERTAIN BACTERIA.

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The presence of lipochromes in bacteria, *i.e.* in plant cells free from chlorophyll, should afford an excellent opportunity for studying both the conditions of formation and the functions of these substances, which, so far as chemical examination has shown, appear to be highly coloured, rather unstable hydrocarbons or simple derivatives.

Zopf has published a series of papers [1889, 1, 2; 1892] in which he has described the "chromolipoids" in eight species of bacteria, a summary of his work being given by Palmer [1922]. In three organisms, viz. *B. egregium*, *B. chrysogloia* and *M.* (*staph.*) *aureus*, Zopf recorded fairly definite evidence for carotene formation, for besides his colour tests with acids and solubilities he found that the alcoholic solutions showed two absorption bands 492–497$\mu\mu$ (covering the F line) and 456–442$\mu\mu$ (between the F and G lines). In *Sphaerotilus roseus* he found a yellow water-soluble pigment and a red alcohol-soluble one, the latter showing the bands of carotene. In the other four cases his evidence is not convincing.

Practically the only other recorded work on the occurrence of lipochromes in bacteria is that of Schrotter [1895], who briefly reported experiments to show that the pigments of *Sarcina aurantiaca* and of *M.* (*staph.*) *pyogenes aureus* give the solubility properties and colour reactions with conc. H_2SO_4 of "lipoxanthin," his general term for a lipochrome. However, he did not study the absorption spectra. Consequently it seemed desirable, in view of the improved technique due to Willstätter and his colleagues [1907–13], Tswett [1906, 1] and others, to examine in detail the pigments produced by some of the coloured organisms.

An additional stimulus to this enquiry lay in the more or less general association of vitamin A with lipochromes in the tissues of higher plants exposed to the light [Steenbock and Cross, 1919; Rosenheim and Drummond, 1920; Steenbock, Sell and Boutwell, 1921; Steenbock and Sell, 1922; Coward, 1923], and the absence of vitamin A but presence of lipochromes in etiolated plant tissues [Coward, 1924, 2]. This suggested the advisability of making an examination of the coloured organisms for the presence of vitamin A.

The two organisms investigated in this work are *Sarcina aurantiaca* and *Streptothrix corallinus*. Both are readily cultivated and are deeply pigmented, but differ in form and method of multiplication.

A. *SARCINA AURANTIACA*.

Large cultures of this organism were grown in Roux bottles on broth agar. After about ten days the growth was scraped off the surface, ground with anhydrous sodium sulphate, and boiled with alcohol for five minutes. Practically the whole of the pigment was thus extracted. Preliminary tests for the general lipochrome reactions were carried out on the material extracted by the alcohol and gave positive results.

Solubility. Insoluble in H_2O. Soluble in alcohol, ether, light petroleum and chloroform.

Colour tests. Greenish-blue colour with conc. H_2SO_4, HNO_3, and I in KI solution.

Kraus' phase test. The alcoholic extract was evaporated to dryness in an atmosphere of nitrogen, dissolved in 5 cc. light petroleum, and an equal volume of 90 % alcohol added. The pigment was almost entirely "epiphasic," so probably little or no xanthophyll was present.

At this stage it seemed evident that probably more than one lipochrome was present, so it was decided to adopt Tswett's chromatographic method of analysis. Saponification of the organisms with alcoholic potash and subsequent extraction with ether and light petroleum did not give satisfactory results, owing to the formation of yellow resinous material during saponification which is also extracted by light petroleum. The method finally adopted was that used by Coward [1924, 1] for higher plant tissues, the massed organisms being saponified with aqueous potash, and 20 cc. alcohol being added immediately before extraction with ether. The process was carried out as far as possible in an atmosphere of nitrogen. The ethereal solution was evaporated to dryness and the unsaponifiable matter redissolved in light petroleum (B.P. 40–60°). The chalk column was prepared according to Tswett [1906, 2] and Coward [1924, 1], and 10 cc. of the light petroleum solution, now presenting a strong pinkish yellow appearance, were used.

Chromatograph of pigment. As soon as the pigment entered the chalk, three separate bands appeared: (*a*) a wide yellow band; (*b*) a sharp yellow band, rather more pink than (*a*); (*c*) a diffuse yellow band at top; but on further washing with light petroleum all three bands travelled downwards and out of the tube, thus confirming the phase test, as the slightest trace of xanthophyll should have persisted as a sharp band in the chalk, even after repeated washing. Fractional filtration through the chalk was now adopted. 25 cc. of the light petroleum solution of the pigment were allowed to pass slowly through the column, and thus divided into 2 cc. fractions, each being examined spectroscopically.

Fraction	Band I	Band II	Band III
I	509–489	478–458	446–
II	508–490	476–458	446–432
III	510–491	475–457	447–435
IV	509–490	477–457	447–435
V	509–489	477–459	447–435
VI	490–475	466–451	448–435
VII	490–474	458–439	—
	—	466–451	—
	—	456–440	—
VIII	490–476	456–440	—
IX	492–475	453–440	—
X	492–475	453–440	—
XI	491–477	458–441	—

Width of slit 0·08 mm. throughout.

If fractions VI and VII are discarded as mixtures, these figures seem fairly clear evidence that the pigment is a mixture of lycopin and carotene. Compared with results of other observers, the separation seems satisfactory.

Lipochrome	Band I	Band II	Band III	Observer
Lycopin	509–490	477–458	447–435	Average fractions I to V above
,,	509–492	479–461	447–435	Coward [1924, 1]
,,	507–490	476–457	443–433	,, ,,
,,	510–499	480–468	440 to end	Willstätter and Escher [1910]
Carotene	491–476	456–440	—	Average VIII to XI above
,,	489–471	456–441	—	Coward [1924, 1]
,,	492–475	460–445	—	Tswett [1911]
,,	488–470	456–438	—	Willstätter and Mieg [1907]
,,	490–475	455–445	430 to end	Kohl [1902]

Large quantities of the organism were then grown, saponified and extracted as above, and an attempt made to get crystal formation by approximately separating the lycopin and carotene by means of the chalk column, evaporating each solution to dryness in an atmosphere of nitrogen and redissolving each residue in alcohol to which a little CS_2 had been added, or in light petroleum.

(*a*) Crystals closely resembling lycopin were obtained on two occasions, once from alcohol and CS_2, as clusters of needles growing longer on keeping in the dark, and once from light petroleum, as long prisms with fish-tail ends [cf. Willstätter and Escher, 1910, Fig. 5].

(*b*) The solution of carotene was much less concentrated than that of lycopin, and so far no crystals have been obtained.

Since lipochromes were being formed continuously by these lower plant cells, it was decided to study the effect of conditions of growth on pigment formation. Numerous agar slopes were prepared from the same sample of broth, and then inoculated from the same parent culture.

I. *Light.*

(*a*) 24 slopes in ordinary test-tubes incubated at 22°.

(*b*) 24 slopes in black test-tubes with a close-fitting dark cap over the cotton-plug, but allowing free access to the air, incubated at 22°.

The growth and pigmentation per slope appeared constant throughout, diffuse daylight apparently being quite without effect; next an attempt was made to compare more accurately the ratio of pigment formed per gram of organism in the dark with that in the light. Roux bottles were used and the amount of growth per bottle in a given time was approximately the same in the two cases. The temperature throughout was 20°. As more flasks were used for the dark experiment, the results will be expressed as three fractions as follows:

$$(1)\quad \frac{\text{Total weight of growth in flasks in light}}{\text{Total weight of growth in flasks in dark}} = \frac{4{\cdot}68}{11{\cdot}3} = 0{\cdot}40;$$

$$(2)\quad \frac{\text{Weight of unsaponified matter from material in light flasks}}{\text{Weight of unsaponified matter from material in dark flasks}} = \frac{0{\cdot}041}{0{\cdot}117} = 0{\cdot}35;$$

(3) By Hellige colorimeter.

$$\frac{\text{Amount of pigment from material in light flasks}}{\text{Amount of pigment from material in dark flasks}} = \frac{26}{65} = 0{\cdot}40.$$

For (2) and (3) the organisms were saponified with aqueous potash and extracted as for chromatograph work.

Considering the experimental difficulties and the number of processes involved, one may consider the above figures in good agreement.

In all these experiments diffuse daylight was used; but when cultures of *Sarcina aurantiaca* were exposed to direct sunlight, growth was poor and no colour developed [cf. Hefferan, 1904]. Even old cultures which appeared dense yellow in colour became bleached if placed near a window where the sun could shine on them. Two thicknesses of glass seemed to afford no protection; however, on sub-culturing and removing from the strong light the new growths developed pigment as usual.

II. *Temperature.*

(1) 24 slopes in ordinary test-tubes incubated at 37°.
(2) 24 ,, ,, ,, ,, 20°.
(3) 24 ,, black ,, ,, 37°.
(4) 24 ,, ,, ,, ,, 20°.

At the lower temperature (sets (2) and (4)) growth was much more vigorous than at 37°, but pigment formation per milligram growth was equal in the four cases.

III. *Hydrogen-ion concentration.*

Trypsin agar of varied p_H used throughout.
Temperature 20°.

6 test-tubes on trypsin agar $p_H = 5$ no growth
6 ,, ,, $p_H = 6$ good healthy growth, porcelain-like surface, orange colour
6 ,, ,, $p_H = 7$,, ,,
6 ,, ,, $p_H = 8$ good healthy growth, porcelain-like surface, colour not so deep.

Initial growth seemed slightly more vigorous on the neutral agar ($p_H = 7$), but the total growth and pigmentation after ten days was the same in the tubes at $p_{\mathrm{H}} = 6$, 7 and 8. More accurately graded tubes were used to show the upper and lower limits for growth and pigmentation, the p_{H} being determined by Mrs Kerridge with the glass electrode [Kerridge, 1925].

Pigment appeared even with the slightest growth.
Lowest limit for active growth: $p_{\mathrm{H}} = 5{\cdot}30$.
Maximum growth: $p_{\mathrm{H}} = 7{\cdot}15$.
Highest limit for active growth: $p_{\mathrm{H}} = 9{\cdot}43$.

B. *STREPTOTHRIX CORALLINUS.*

This organism [Reader, 1925] presents a brilliant coral colour when grown at room temperature on agar plates. It was chosen as an example of the higher bacteria, as it was thought that conditions might be different in these organisms from those in a *Sarcina*. The technique used was exactly the same as described above for the *Sarcina*. The general lipochrome reactions all gave positive results, and the alcoholic solution appeared to resemble a lycopin solution closely. It was slightly more difficult to extract than the pigment of *Sarcina aurantiaca*, but previous grinding of the organisms in a mortar with a little kieselguhr overcame this difficulty. When preparing the solution for the chromatograph it was noticed that the light petroleum solution had not the pinkish appearance of the alcohol or ether solutions.

Chromatograph. (*a*) One thin sharp layer travelled slowly down the tube, became irregular and stationary. (*b*) One dense wide band 0·2 inch in depth stayed at the top. On washing with light petroleum it widened very slightly but neither divided nor moved downwards.

The material in fraction (*a*) was dissolved out in ether, but the faintly yellow solution was too weak for spectroscopic examination. Fraction (*b*) was dissolved out in ether, and evaporated down slightly. On spectroscopic examination this solution showed two well-marked absorption bands.

	Band I	Band II
Strong	509–485	465–455
	505–485	465–453
	508–486	465–454
Weaker	505–485	466–454
	507–485	467–455

Width of slit 0·08 mm. throughout.

Neither light petroleum of B.P. 40–60°, nor of B.P. 60–80°, caused this pigment to be washed through the chalk column as lycopin would be.

As it was possible that the position of the bands had been shifted somewhat as a result of the saponification, the following comparisons were made:

	Band I	Band II
Direct extraction with hot alcohol	508–485	466–453
	507–485	465–454
After saponification	508–485	456–453
	508–485	465–453

A few flasks seemed to have a yellow rather than coral appearance. However, since the extracted pigment from these gave the same absorption bands, probably the effect was due to quantitative rather than qualitative differences.

Band I	Band II
507–482	465–451
508–483	466–454

All attempts to divide this pigment into two parts by fractional filtration, even into as many as ten fractions, were unsuccessful. Hence it was decided that it must be another lipochrome, the absorption spectrum of which had not been previously recorded. The suggested name is "coralin."

The formation of pigment under different conditions was studied and has been reported in detail in another paper [Reader, 1925]. The agar slopes were prepared as for *Sarcina aurantiaca* and arranged in sets as before. It was observed that the cultures at a low temperature (20°) were highly pigmented both in the light and in the dark, but that even after six weeks no pigment had been formed at 37°. A second series more accurately graded showed that maximum coloration appeared at 18°, and that above 27° only traces of colour could be found. Growth was vigorous from 22° to 37°.

Slides were made regularly from every set and stained by Gram's stain. It soon became apparent that the development of pigment was parallel with the segmentation of the bacilli or rods. This is only the case when a broth or other complete medium is used. In synthetic medium (inorganic salts + glucose) there is no pigmentation even at 18°. When trypsin broth or agar is inoculated with this organism, both the segmentation and the development of pigment depend on the temperature, as above 27° the growth is rapid, filamentous, and colourless.

Direct sunlight did not have the effect of decoloration on this organism.

PRESENCE OF VITAMIN A IN THESE PIGMENTED ORGANISMS.

Considerable quantities of these organisms were grown in Roux bottles, saponified with alcoholic potash for 3 hours, extracted with ether, washed with boiled water, dried over sodium sulphate and evaporated nearly to dryness, the whole process being as far as possible carried out in an atmosphere of nitrogen. The unsaponifiable matter was mixed with medicinal paraffin in order to feed it in varying doses to rats which had become steady in weight on a vitamin A deficiency diet. No growth of the rats was obtained; also the colour tests for vitamin A [Rosenheim and Drummond, 1925] were negative. In the case of *Streptothrix corallinus* all the usual tests (Salkowski's reaction, Liebermann's reaction, Tschugaieff's reaction, Neuberg's reaction, etc.) for cholesterol and related compounds were negative. In the light of the recent work of Steenbock [1925] and of Drummond and Coward [1925] on the formation of the antirachitic factor by exposing cholesterol to ultra-violet light, this is interesting. The extract from *Sarcina aurantiaca*, however, gave good sterol reactions.

In a recent publication Luce and MacLean [1925] claimed that the presence of traces of vitamin A in ordinary yeast could be proved if large enough doses of unsaponifiable matter were fed to vitamin A-deficient rats. In this connection one interesting fact has been noted in my preliminary extractions of unsaponifiable matter of large cultures of yeast. A concentrated solution of this extract was yellow in colour and gave definite carotene absorption bands when examined spectroscopically in both chloroform and ether solutions. Observations in these solvents by Kohl and by Coward are recorded for comparison.

Solvent	Band I	Band II	Observer
Chloroform	505–480	465–450	Kohl [1902]
,,	505–480	465–450	Reader
,,	506–483	465–450	,,
,,	506–483	465–450	,,
Ether	490–475	455–445	Kohl [1902]
,,	491–476	456–440	Reader
,,	489–471	456–441	Coward [1924]

This unsaponifiable matter from yeast does not give the vitamin A colour tests with dimethyl sulphate, arsenic trichloride or trichloroacetic acid (50% solution in chloroform), so possibly it is only the antirachitic factor which is formed, for Steenbock [1925] has shown that this factor is frequently found in the absence of the growth-promoting fat-soluble vitamin. Using two methods of testing for the presence of antirachitic activation, viz. (*a*) the induction of growth and calcification in the rat during phosphate inhibition and (*b*) the cure of rickets produced by deficient phosphate and excess of calcium in the diet, he found that a large number of naturally occurring materials can be activated antirachitically by exposure to ultraviolet light. Among these are yeast, commercial casein, ox bile, lanolin and grains, and their commercial products such as starch, meals, flours, breakfast foods and oils.

SUMMARY.

(1) *Sarcina aurantiaca* contains two lipochromes, carotene and lycopin, the latter predominating.

(2) *Streptothrix corallinus* contains one lipochrome, apparently distinct from the well-known ones, for which the name "coralin" is suggested.

(3) Neither of these organisms forms appreciable quantities of vitamin A, even under the most favourable conditions as regards growth and pigment formation.

(4) Yeast contains traces of carotene.

(5) Some of the higher bacteria, *e.g.* *Streptothrix corallinus*, appear to contain no cholesterols or phytosterols.

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CLI. STUDIES ON THE CHEMICAL NATURE OF VITAMIN A.

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EFFORTS to isolate and to determine the nature of the substance known as vitamin A received a great impetus when it was definitely established that the compound may be concentrated into that part of such substances as cod-liver oil which resists saponification [Steenbock and Boutwell, 1920; Drummond and Coward, 1921][1]. Owing to the instability of the vitamin in the presence of substances tending to produce oxidation it is usually necessary, if loss is to be prevented, to carry out the saponification and extraction in an atmosphere of an inert gas such as nitrogen. When this precaution is taken, it is a comparatively simple matter to concentrate with little or no appreciable loss the vitamin A present in a litre of cod-liver oil into the 8 or 9 g. of unsaponifiable matter that this yields. Furthermore it has been demonstrated that this preparation also contains the whole of the antirachitic vitamin, or vitamin D, of the raw oil [Zucker, Pappenheimer and Barnett, 1922; Zucker, 1922]. The value of concentrating these physiologically important substances may be appreciated from two standpoints. In the first place it is obvious that there is a definite, if limited, need in clinical medicine for a preparation containing in highly concentrated form the components of the oil that are responsible for its therapeutic action; indeed, at least two patents have been taken out and are being operated for the technical preparation of such material for medicinal use. On the other hand the possession of a concentrated fraction, obtainable in reasonably large amounts, provides some encouragement for the attempt to isolate the active constituents.

During the last five years we have been concentrating our efforts in this field, without, we regret to say, any success worthy of note. Our reasons for publishing what is admittedly an incomplete study are two: in the first place, we feel that unless very much larger quantities of raw material (unsaponifiable matter) are available than we have hitherto possessed, the technique by which we have attacked the problem in the past is quite inadequate to carry us much further than we have already gone. Secondly, we desire to give our reasons for challenging a number of claims that have recently been advanced, in particular those of Takahashi [1922, 1923, 1925] and his colleagues, to have isolated vitamin A in pure condition.

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[1] As far back as 1914 McCollum and Davis reported that the growth-promoting vitamin in butter fat withstood saponification [1914]; although later work has shown this to be true, the results of their experiments could not be claimed as being conclusive evidence on this point.

Preparation of the unsaponifiable matter for the study of vitamin A.

The preparation of the concentrate which has been used by us as the raw material for our fractionations scarcely needs full description now since several processes have been given in detail, either in papers or patent specifications, by other investigators. Several points may, however, be emphasised as possibly being of help to others wishing to repeat this work. In these studies simple saponification of the cod-liver oil with potassium hydroxide in alcohol has been exclusively employed. As a second saponification is always necessary it is preferable to make the initial treatment with alkali as short as possible, in order to minimise both inactivation of the vitamin and formation of resinous material from the reagents. The oil is slowly poured with vigorous stirring into the hot solution of the alkali in alcohol; a 10 % excess over the theoretical quantity of potassium hydroxide being ample. When all the oil[1] has been added, the mixture is heated to boiling for about 30 minutes, cooled, diluted with 10 volumes of water, and the unsaponifiable matter extracted with ether. An examination of the product yielded by this initial saponification reveals the fact that practically always a considerable proportion of matter still present can be hydrolysed. It is therefore advisable to submit it to a second saponification, for which it has been found preferable to employ sodium ethylate. It is important that a stream of nitrogen should be passed through the apparatus while this saponification is in progress, for it has been our experience to find that considerable loss of vitamin A may take place at this stage of its extraction, unless great care be taken to prevent contact with air. It is scarcely ever necessary to resort to a third treatment with alkali, the action of the sodium ethylate being sufficient not only to resolve the glycerides which have survived the initial process, but to hydrolyse the fatty acid esters of cholesterol and other higher alcohols present.

The importance of obtaining a product entirely free from esters is considerable if it is desired to submit it to fractional distillation, for we have observed that the presence of small amounts of such substances tends to give rise to more decomposition during the distillation at low pressures than is desirable. No material was employed by us for further treatment until a test carried out on a small sample showed that saponification had been complete. There appears no doubt that the method of saponification employed by Zucker [1922], and by Takahashi [1922], in which a large proportion of the fatty acids are removed in the form of the relatively insoluble calcium or barium soaps is more advantageous than the rather tedious process we adopted, although the yield of unsaponifiable matter appears to be slightly larger in our case. We have found ethyl ether to be the most convenient solvent for the extraction of the unsaponifiable matter from the alkaline solutions of the soaps, but its suitability as an agent of extraction is lessened to some extent by the difficulty of obtaining it pure in large quantities. The problem of extracting the active material from the products of hydrolysis of some hundredweights of cod-liver oil is not an easy one, even when the laboratory is equipped with small scale "plant," and the choice of an extracting agent is particularly difficult. The employment of ether may lead to a variety of troubles, of which the accumulation of the less volatile impurities usually present in the solvent —mainly mixed ketones of the type of methylpropyl ketone—in the final product is less disturbing than loss of material and damage caused by an explosion due to the accumulation of so-called "ether peroxide." Some experience of these troubles has led us to investigate a number of other solvents, particularly the inert hydrocarbons, but the latter tend to give rise to troublesome emulsions, and we have not yet found a solvent satisfying our requirements. Meanwhile we have continued to employ ether. The yield of material obtained from the initial hydrolysis of ordinary samples of cod-liver oil is usually from 1·5–2 %, and this is reduced by further saponification to about 0·8 %. The product is a semi-solid crystalline mass with a waxy appearance and varying in colour from pale golden yellow to deep orange according to the colour of the original oil. A brown or brownish red colour usually indicates that the production of resin during the saponification has been unnecessarily great. The crystalline appearance can readily be recognised as being due to flat prisms of cholesterol. Tested for the presence of vitamin A either by feeding tests with rats or by means of the colour reactions recently described [Rosenheim and Drummond, 1925] this preparation, if made and stored with due precautions against oxidation, is found to be equivalent to the oil from which it was extracted.

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[1] In our earlier investigations we employed a pale Norwegian oil of high technical quality. Later, as the result of other investigations [Zilva and Drummond, 1923], we found that the vitamin A value of oils from Newfoundland is usually many times greater than that of Norwegian oils from the Lofoten area, and, accordingly, we employed as far as possible oils from the Dominion as our raw material. We are indebted to Mr W. A. Munn and Mr Davies of St John's, Newfoundland, for supplies of oils from this source.

CHEMICAL EXAMINATION OF THE UNSAPONIFIABLE FRACTION OF COD-LIVER OIL.

Iodine and Nitrogen.

An examination of the unsaponifiable matter revealed that the constituents were entirely organic in nature, and that the only recognisable elements present were carbon, hydrogen, and oxygen. Very careful tests were made to detect any trace of iodine or nitrogen, because of the still lingering theories that these elements are connected with the physiological activity of the oil. Nearly a century ago Hupfer de l'Orme [1836] first detected the presence of iodine in cod-liver oil and suggested that the therapeutic action was due to traces of that element. At first the theory was coldly received and even the presence of iodine was doubted [Gmelin, 1837; Potemka, Martius and Sarphati, 1838], but when it had been established definitely that this halogen was usually present in the oils then on the market [Springmüll, 1837; Gmelin, 1838; Bennett, 1841], the theory became widely accepted. Indeed it was almost generally held until Williams [1912, 1913] pointed out that the therapeutic action of cod-liver oil is unrelated to its iodine content and suggested as an alternative theory that it is probably due to the readiness with which the highly unsaturated fatty acids present in cod-liver oil might conceivably be assimilated by the organism. Until recent years, the fatty acid theory and the iodine theory may be said to have shared popularity. The demonstration that the therapeutic action of cod-liver oil can be transferred to the unsaponifiable fraction and that the fatty acids themselves are without action [Drummond, 1919] rendered the former hypothesis no longer tenable. The iodine theory survived, however, because it was possible that the active complex containing iodine might be concentrated into the unsaponifiable matter, until it was shown that there are no detectable traces of the halogens in the active fractions [Drummond and Coward, 1922]. This we have many times confirmed ourselves, and it has been established also by an independent test kindly carried out for us by Dr Harington on a relatively large quantity of a highly active product. In view of these observations, as well as of the fact that the medicinal value of cod-liver oil cannot, as far as our experience goes, be correlated with its iodine content, we feel that there are no adequate reasons for the recent suggestions that it may be necessary to revive the iodine theory. The absence of all traces of nitrogen from the unsaponifiable fraction, when carefully prepared, demonstrates that the substances responsible for the therapeutic action of the cod-liver oil do not contain this element. The work of Funk [1915, 1916] in which processes are described for the isolation of vitamins from cod-liver oil, may be dismissed because the methods could only be concerned with the extraction of substances of the type of nitrogenous bases. This investigator must however, be given credit for being the first to recognise that the curative value of cod-liver oil in rickets and wasting diseases is probably due to one of the substances then so recently discovered by Hopkins [Funk, 1914].

Cholesterol.

As far back as 1886 Salkowski recognised the presence of cholesterol and of a lipochrome, not carotene, in the unsaponifiable fraction of cod-liver oil [1886]. He proved the identity of the cholesterol satisfactorily and showed that the slight laevo-rotation exhibited by cod-liver oils can be accounted for by the presence of this substance. His observations have been confirmed by us. It has been shown previously [Drummond and Coward, 1922] that the cholesterol may be quantitatively removed by crystallisation and precipitation with digitonin without removing vitamin A, and this observation has been confirmed many times by ourselves and by other observers [Takahashi and Kawakami, 1923].

Removal of Cholesterol.

Estimations of cholesterol by the digitonin method in a large number of samples of unsaponifiable matter from cod-liver oil give values ranging from 47·8 to 50·9 %. The majority of values are close to 50 %. The removal of this constituent is best effected in our opinion by the following process. The weighed material is treated with twice its weight of boiling methyl alcohol, nitrogen being bubbled through the solution, and the orange yellow liquid is then cooled in ice. Approximately 90 % of the cholesterol present separates and may be removed by filtration, preferably conducted in an atmosphere of nitrogen. After repeated crystallisation from alcohol, acetone and light petroleum, the cholesterol shows M.P. 148·5–149° (corr.), and gives acetate, M.P. 114·3°, benzoate, M.P. 145°; and has $[\alpha]_{\mathrm{F}}^{15^\circ}$, − 40°. It is inadvisable to cool the liquid much below zero in separating the cholesterol because other constituents tend to crystallise out at lower temperatures. The removal of the residual cholesterol can only be satisfactorily carried out by the method of precipitation with digitonin introduced by Windaus. The great expense of this reagent and the difficulty of obtaining it in quantity have proved the main factors limiting our studies. The mother liquors from the crystallisation of the cholesterol are evaporated at low pressure to remove the methyl alcohol, and any further cholesterol separating during the concentration is removed; if the cooling of the original solution has been prolonged, there is usually little or no further separation. For the preparation of material for therapeutic use, it is, of course, unnecessary to remove the cholesterol entirely, and the examination of several products at present on the market has shown that only the preliminary separation by crystallisation from a suitable solvent has been employed. Such preparations usually contain from 5 to 9 % cholesterol. The removal of the remaining cholesterol is carried out by dissolving the material in four volumes of 95 % alcohol and adding the necessary excess of a 1 % solution of crystalline digitonin (Merck) in boiling 90 % alcohol; the solution is maintained just below boiling point while a slow current of nitrogen is passed continuously through it. After tests have shown that the precipitation of the cholesterol digitonide is complete, the mixture is allowed to stand overnight at room temperature, and the digitonide is removed on the following morning by filtration. The precipitate must be thoroughly washed with 95 % alcohol and finally with a little ether. The filtrate with the added washings is evaporated at low temperature and pressure to remove the solvents, the residue dissolved in ether, and the small excess of digitonin washed out by repeatedly shaking the ethereal solution with water in an atmosphere of nitrogen. Finally the ethereal solution is separated and dried with anhydrous sodium sulphate and the solvent removed at low pressure. The product thus obtained is a deep reddish orange oil possessing the characteristic smell of the original unsaponifiable fraction. If it is carefully prepared it shows a vitamin A value proportionate to the cod-liver oil from which it was prepared. The cholesterol, whether prepared by purification of the material separating from the methyl alcohol solution, or from the digitonide by decomposition with hot xylene vapour in an inert atmosphere, shows no physiological activity. This confirms our earlier observations on the inactivity of pure cholesterol from other sources, *e.g.* brain [Drummond, 1919].

Fractionation of the Cholesterol-free Residue.

A superficial examination of the viscous oil which remained after cholesterol had been removed suggested that it was to a large extent composed of substances of the nature of unsaturated alcohols. Accordingly, efforts were made to separate the constituents by means of fractional distillation. A brief outline of our earlier results was communicated to the Biochemical Society at the meeting on May 10th, 1924 [Drummond and Coward, 1924], in which it was shown that vitamin A is volatile in superheated steam at 115–125°, and that by fractional distillation at low pressure it could be concentrated in a fraction boiling at about 250° (3–4 mm.).

Distillation in Superheated Steam.

As a method of effecting any further concentration of vitamin A we soon abandoned this method, for not only is it very tedious, but it effects no separation of the constituents other than the removal of the non-volatile resins derived from the saponification. The distillation must be carried out in an atmosphere of nitrogen a rapid stream of which must sweep through the apparatus before distillation commences, as well as throughout the experiment. The superheated steam slowly carries over the volatile constituents of the oil, the distillate condensing as an emulsion of semi-solid consistency. To avoid temporary blocking of tubes by the firm candle-like emulsion it is advisable to fit the apparatus with a small inlet tap, whereby drops of ether can be allowed slowly to fall into the upper part of the condenser during the distillation. By this means the emulsion is broken down so that distillation proceeds without any interference. The process is very slow indeed, and it requires many hours to distil over 10–20 g. of the oil. The distillate, after separation from water, is a pale lemon yellow coloured oil possessing the characteristic terpene-like smell of the original material. It gives in undiminished intensity the colour reactions to which reference has been made, and on administration to rats proves as potent a source of vitamin A as the equivalent amount of original oil or unsaponifiable matter. The yield of active distillate corresponds to from 0·3 to 0·4 % of the original oil. The distillation residue, after dehydration, is a dark brown, amber-like gum, corresponding in weight to about 0·05 % of the original oil. It gives no response to the colour reactions, and is inactive when tested on animals.

Distillation of the Cholesterol-free Fraction under reduced pressure.

During the course of this work a large number of fractional distillations of the cholesterol-free fraction of the unsaponifiable matter from cod-liver oil have been carried out. The earlier experiments indicated that to effect any sort of separation of the constituents by this means would be no simple matter, primarily because the range of temperatures over which the fractionation is conducted is short. Therefore it was necessary to distil a reasonably large volume of liquid, to employ a fractionating column of suitable design and length, and carefully to maintain control of the temperature difference between the boiling and distilling fluid. With these precautions, and a series of refractionations, it was hoped to separate the chief constituents in a state sufficiently pure to enable one to determine their nature. Unfortunately, the work throughout has been hampered by the great difficulty of obtaining sufficient material to enable a satisfactory fractionation to be performed. In the earlier investigations quantities of from 20 to 50 g. of the cholesterol-free oil were frequently distilled. These preliminary trials, unsatisfactory as they were, gave us some indication of the results we might expect on a large scale experiment. Working at pressures of from 2–4 mm. we usually collected the distillate in four fractions [Drummond and Coward, 1924].

I. A very small fraction of a clear colourless liquid distilling over the range 40–70° (4 mm.). II. A clear, slightly yellow oil collected between 180–220° (3 mm.). III. A clear, pale yellow oil 220–260° (3 mm.). IV. A heavy, golden yellow oil 260–300° (3 mm.). The residue remaining behind in the flask was a very heavy reddish brown oil showing a marked green fluorescence. On cooling, it solidified to an amber-like resin. Of the volatile fractions, we have ignored the first on the ground that it is almost certainly derived from the ether used in the extraction. Owing to the small amount that has been available we have not succeeded in identifying it. It usually possessed an iodine value of the order of 20, indicating a saturated compound contaminated with traces of the unsaturated compounds in the higher fraction. Its odour resembled that of the higher ketones. Attempts to prepare a crystalline derivative failed. It had no effect on growth. Indirect light on its nature was obtained during some experiments on the reduction of certain fractions from the unsaponifiable matter with hydrogen in the presence of catalytic nickel. In these experiments a liquid of relatively low boiling point distilled over from the hydrogenation vessel. Its smell closely resembled that of octyl alcohol, and as far as the small amount available permitted us to establish its identity the belief that it was one of the octyl alcohols was confirmed[1]. Our earlier studies of the substances present in the other fractions II, III and IV, led us to believe that they were largely composed of unsaturated alcohols [Drummond and Coward, 1924; Drummond, 1924], but the amounts obtained were quite inadequate to enable us to determine their nature. A few details may be given of a typical distillation of this type. 69 g. of unsaponifiable matter—representing about 10 litres of a light coloured Lofoten oil—yielded a total of 33 g. of cholesterol and just over 30 g. of the orange coloured oil. This was separated from resins by distillation in a current of superheated steam and nitrogen, and yielded 25 g. of a clear, pale yellow oil. On distillation at 3 mm. pressure this oil yielded the following fractions:

(*a*)	100°	0·20 g.
(*b*)	100°–180°	1·2
(*c*)	180°–230°	8·29
(*d*)	230°–240°	1·40
(*e*)	240°–260°	6·23
(*f*)	260°–300°	1·73
Undistilled residue		3·5
		22·55 g.

It must be stated, however, that on many occasions the rate of distillation from 180° to 300° was steady. In the experiment described it was only possible to attempt redistillations of fractions (*c*) and (*e*), and these yielded respectively 5 g. of a clear lemon-yellow coloured oil boiling between 200° and 220°, and 3 g. of a darker yellow oil boiling at 250–260° at 2–3 mm.

Vitamin A Content of Fractions.

The fractions (*b*)–(*f*), as well as the undistilled residue, were tested for vitamin A by feeding tests. These indicated that vitamin A passed over mainly between 180–220°, at 2–3 mm. These experiments conclusively proved that the vitamin is volatile at these temperatures (3 mm.), and that very little loss of activity takes place during the operation[2]. It may be said, in passing, that throughout this research we have noted a perfect agreement between the intensity of the colours yielded by the fractions on applying the sulphuric acid test [Drummond and Watson, 1922] or the later substitutes for that test [Rosenheim and Drummond, 1925] and the activity of the fractions as determined by feeding experiments on rats.

[1] The occurrence of impurities, mainly mixed ketones, derived from the ether has been a frequent source of trouble to us. We first fully appreciated its disturbing effect when engaged in the fractionation of a large batch of unsaponifiable matter. During the extraction of this material we concentrated in our product the less volatile of the impurities of approximately 200 l. of ether. At later stages in the separation of the active fractions we obtained a total of nearly 50 cc. of mixed ether impurities. Fractionation of this material yielded four main fractions, all possessing the typical smell of the higher ketones. The presence of ethylmethyl ketone, B.P. 81°, diethyl ketone, B.P. 102°, and another ketone, B.P. 130°, was indicated by an examination of these fractions.

[2] Curiously, we have experienced rather serious losses of activity during the refractionation of the initial fractions, in spite of all attempts to conduct the later distillations with as great, or even greater, care. As yet we have no satisfactory explanation of this occurrence.

Preliminary Chemical Examination of Main Fractions (c) *and* (e).

Although it was obvious that the redistilled fractions (*c*) and (*e*) were probably impure, they were submitted to a superficial examination in order to ascertain something of their nature. The results of this examination indicated, as we had expected, that the chief constituents were unsaturated alcohols. Thus the averages for a number of determinations of the iodine value on various samples of each product were: (*c*) 110, (*e*) 130. The corresponding acetyl values were (*c*) 170, (*e*) 164, and the approximate molecular weights, (*c*) 320, (*e*) 350; neither fraction was optically active. With the sure knowledge that our products were impure, it was obviously unsafe to attach more importance to these data than merely regarding them as indications that unsaturated alcohols probably formed a considerable proportion. Certainly it would have been absolutely unjustifiable to regard either one of these preparations as the vitamin A in even approximately pure condition.

Detection of an Unsaturated Hydrocarbon in Cod-liver Oil.

In the preliminary study of these fractions it was ascertained that on treatment with an excess of bromine in anhydrous ether solution a slow deposition of a heavy white microcrystalline compound occurred. The manner in which this substance was precipitated and its general appearance suggested a similarity to the ether-insoluble dodecabromide of the curious unsaturated hydrocarbon that has been found in liver oils from other species of fish. This hydrocarbon has been termed squalene by Tsujimoto [1916, 1920] and given the formula $C_{30}H_{50}$, whereas Chapman [1917, 1918, 1923] believes it to be represented by $C_{29}H_{48}$ and calls it spinacene. The bromide from the cod-liver oil fractions is almost insoluble in dry ether (0·11 % at room temperature), and may be recrystallised in a rather unsatisfactory manner by dissolving in tetrachloroethane, in which it is fairly soluble, filtering and reprecipitating by the addition of alcohol or dry ether. There remains little doubt that the bromide prepared from the cod-liver oil fraction is identical with the bromide of spinacene $C_{30}H_{50}Br_{12}$. Estimations of bromine by Carius' method gave values of 68·57 %, as compared with 70·07 % required for the formula $C_{30}H_{50}Br_{12}$, or 70·80 % required for $C_{29}H_{48}Br_{12}$[1]. It shows no definite melting point, but on heating darkens at about 160°, and decomposes without sharp melting at 180–190°. The presence of spinacene in our fractions was confirmed by the preparation of the typical hexahydrochloride by passing dry hydrochloric acid through the solution of the substance in dry ether or dry ethyl alcohol until it is saturated. All our fractions yielded a precipitate of the characteristic crystalline hydrochloride, which can be readily obtained in a pure condition by recrystallisation from acetone. A large number of preparations from our fractions showed close resemblance to the hydrochloride prepared from specimens of pure spinacene kindly provided by Mr Chaston Chapman, F.R.S. Melting point varies from 110–125°, chlorine content 33·70,

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[1] Owing to the difficulty of purifying the bromide we have failed to get figures for the bromine content close to the theoretical, even when analysing the product prepared from pure spinacene.

33·52 %. Calculated for $C_{30}H_{56}Cl_6$, 33·86 %; $C_{29}H_{54}Cl_6$; 34·63 %[1]. The detection of spinacene in all our fractions, and our inability to separate it from the other components by refractionations, impressed us with the difficulties which faced us in our efforts to determine the nature of the vitamin A. Indirect evidence that this hydrocarbon was not responsible for the effect on growth was provided by the fact that the fractions of highest boiling point tended to be richest in spinacene, but of relatively low vitamin value. Direct tests on a sample of unsaponifiable matter containing approximately 85 % of spinacene, for which we are indebted to Mr Chapman, as well as on a pure specimen made by us from the liver oil of *Spinax niger*, confirmed this view. Spinacene yields no response to the colour reactions we have referred to.

Separation of a Solid Saturated Alcohol (*Batyl alcohol?*).

It was observed that a small amount of a crystalline substance separated from the fractions of higher boiling point on standing for several days. On removing the crystals and recrystallising from acetone or methyl alcohol, a very small amount of a clear white product was obtained. There was just sufficient material to serve for a feeding test on two rats, which demonstrated its inactivity. The substance melted at 60°, was saturated, and was, we think, the same substance as is described below.

A solid substance closely resembling the product from cod-liver oil was isolated from the liver oil of *Spinax niger*. The unsaponifiable matter represents some 80 % of this oil and is almost entirely composed of spinacene. Purification of the spinacene by treatment with methyl alcohol, in which it is very slightly soluble at 0°, yields a fraction of more soluble material from which the crystalline substance separates in appreciable amounts. On recrystallisation from methyl alcohol we have obtained a preparation with a melting point as high as 65°. The Japanese investigators give the melting point of batyl alcohol as 69°. The crystalline form resembles that of the compound obtained from cod-liver oil[2].

Further manipulation of our fractions gave no satisfaction. We were quite unable owing to the very small amount of material available to isolate any other substances or their derivatives in the pure condition.

A consideration of our progress up to this point made us realise that only two paths were open by which further advances could be made. One would be to repeat our previous work on a much larger scale, hoping thereby to obtain fractions suitable for repeated refractionation, whereas the other would be to gather indirect evidence as to the nature of vitamin A by the examination of the unsaponifiable material in certain other fish-liver oils. Both these paths have been traversed during the last two years with, we fear, indifferent success.

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[1] The melting point of the hydrochloride is uncertain. Repeated recrystallisation from hot solvents causes a diminution in chlorine content. The substance twice recrystallised from hot acetone usually melts about 115°.

[2] Four analyses of the solid alcohol gave figures close to the averages of C 72·29 %; H 12·48 %. Batyl alcohol, if it possesses the formula $C_{20}H_{40}O_3$ requires C 72·7 %; H 12·7 %.

Large Scale Fractionation of Unsaponifiable Material from Cod-liver Oil.

We originally intended to prepare material for this study by saponifying a large quantity of cod-liver oil on a small technical scale. Mr E. R. Bolton, of Technical Research Works, Ltd., undertook to prepare material in his experimental plant according to our directions. A certain amount of oil was treated in this manner, but, in spite of very considerable care, it was found that rather serious loss of vitamin activity had occurred during the process. As opportunity for investigating the cause of the inactivation did not immediately present itself, we fell back on a large-scale laboratory preparation in which 5–7 litres of oil were saponified at one time. We wish to express our indebtedness to Mr Bolton and his co-worker, Mr Lush, for the help and advice they so generously gave.

The collection of unsaponifiable material by the alternative method was somewhat tedious, and we suffered the disappointment of losing a considerable proportion of the total of more than a kilogram owing to an "ether peroxide" explosion in the final stage of preparation. Sufficient material was saved, however, to enable us to carry out a separation on a much larger scale than had previously been attempted. The general lines along which the separation was conducted have been already described. 750 g. of unsaponifiable matter yielded a total of 370 g. of cholesterol (49·3 %). It was during the concentration of the cholesterol-free fraction of this preparation that nearly 50 cc. of mixed impurities of ether were obtained (see footnote, p. 1054). The fractionation under reduced pressure of a portion of the cholesterol-free oil was carried out with great care, employing a fractionating column and adequate "lagging." The initial fractionation yielded just over a gram of a clear liquid boiling below 40° at 3–4 mm. This fraction was apparently identical with that described on p. 1053, which we believed to be a C_8 ketone, derived from the reagents employed in the extraction. The distillation of the main bulk of the oil failed to give us a well-marked separation, being fairly regular throughout after 150°.

The following fractions were collected and on examination gave the data:

Fraction	Description	Temperature Range	Quantity g.	Iodine value	Acetyl value	Colour reaction[1]
II	Very pale yellow oil, marked terpene-like odour	125°–155° (3 mm.)	5	114	·	– } + }
III	Pale yellow oil, terpene odour	155°–184° (2 mm.)	7	120	196	+ +
IV	Yellow oil, similar smell	184°–190° (2 mm.)	6	125	120	+ + + +
V	Yellow oil, pungent terpene-like smell	190°–220° (1–2 mm.)	36	129	174	+ + + +
VI	Orange yellow oil, pungent smell	220°–270° (1–2 mm.)	27·5	149	140	+ +
VII	Reddish brown amber-like resin	Residue 270°	20·5	·	·	–

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[1] We invariably employed the colour reactions as a preliminary indication of the presence of vitamin A, reserving the animal tests for the examination of the more important fractions. On no single occasion in the course of five years' work has this procedure misled us.

Attempts were made to refractionate V and VI, but without any real success, and we were reluctantly forced at length to conclude that, without possessing very much larger quantities of material, there was little hope of separating the constituents satisfactorily by this means. A considerable amount of confirmation of our earlier results was, however, obtained by an examination of the redistilled fractions V and VI. Spinacene was present in both fractions, but to a considerably greater extent in that of higher boiling point, which also yielded an appreciable amount of the solid, saturated alcohol (M.P. about 60°) referred to previously. Sufficient of the substance was not obtained to purify it adequately, much less to establish its nature, but we have reasons for believing that it is identical with the substance described on p. 1056 as being isolated from the liver oils of the fish, *Spinax niger*. We were able to confirm its inactivity when tested on animals.

Attempted Fractionation by means of Solvents.

Having failed to separate the constituents of V and VI by distillation, we turned to fractional solution as a means of achieving our aim. These oils are extremely soluble in most fat solvents, ether, light petroleum, chloroform, carbon disulphide, carbon tetrachloride, tetrachloroethane, trichloroethylene, but are less soluble in methyl and ethyl alcohols and acetone. Takahashi and Kawakami [1923] reported that the cholesterol-free fraction of cod-liver oil unsaponifiable matter yields on recrystallisation from 80–90 % methyl alcohol at − 20° a semi-crystalline substance, which they regarded as vitamin A in the nearly pure condition[1]. We repeated their experiments, testing not only methyl and ethyl alcohol but also acetone as a solvent. Slow crystallisation at temperatures about − 10° throws down a flocculent, white precipitate, which, seen under the microscope, consists of bunches of slender needle-shaped crystals mixed with fine droplets of oily substances. The crystals melt about zero, and a pale yellow oil is produced. This product was examined chemically, as well as for vitamin activity. As we reported some time ago, it is certainly not vitamin A [Drummond and Coward, 1924] because it is less active in promoting growth than the mother liquors. Furthermore, the material thrown down from solution on cooling tended to be decidedly richer in spinacene than the original fraction, as indicated by the iodine values and the yields of insoluble bromide on treatment with bromine. The application of these processes of crystallisation did not seem to facilitate the purification of any of the constituents present in our fractions, and we abandoned them. We also attempted to effect some separation by means of differential solubilities in mixtures of solvents. Our exploration in this direction was very superficial, being limited to an examination of the so-called "phase test," frequently employed in the separation of the lipochrome pigments. 2 g. of an active fraction were dissolved in 20 cc. of light petroleum and shaken up vigorously at room temperature with an equal volume of methyl alcohol. On separation of the liquids, of which the light petroleum was distinctly the more deeply coloured, and removal of the solvents, two fractions were obtained. The fraction soluble in methyl alcohol weighed 0·48 g. It possessed an iodine value of 116, gave a strong colour reaction, and produced growth in the test rats. The light petroleum fraction weighed 1·42 g., had an iodine value of 129, and was of definitely higher vitamin value.

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[1] This claim to have isolated vitamin A is an earlier one than that criticised on p. 1062. The active substance was then believed to be an aldehyde.

At the time the results did not appear to be particularly encouraging, and the experiments were discontinued, but it is not unlikely that it would be worth while making more careful studies in this direction.

Nature of the Unsaturated Alcohols Present.

The results obtained seemed to indicate that apart from spinacene, the saturated alcohol (M.P. about 60°), and possibly unchanged lipochrome pigment, the bulk of the cholesterol-free fraction consisted of one or more unsaturated alcohols. In our earlier studies we were inclined to believe, from the progress of the fractional distillations, that two such alcohols were present, one boiling about 210° (3 mm.) and the other about 250° (3 mm.) [Drummond and Coward, 1924]. Later work, in which larger quantities of material were employed, has tended to weaken this view, and our inability to obtain any satisfactory fractionation leads us to believe now that probably only one unsaturated alcohol is present in any appreciable quantity. Our examinations of the fractions for this substance have been greatly impeded by the difficulty of removing the spinacene, or of forming a trustworthy opinion as to the amount of that hydrocarbon present. An attempt was made to estimate the amount of spinacene present by weighing the yields of dodecabromide and hydrochloride. Unfortunately, our experience leads us to confirm the view shared by Chapman and by Tsujimoto that both reactions proceed abnormally and that they cannot be regarded as quantitative. Of the two reactions, that of the formation of the hydrochloride is without doubt the more reliable. A series of estimations of the yield of this compound obtained from specimens of practically pure spinacene indicates that the yield of hydrochloride depends on the concentration of the hydrocarbon in the solution. In dry ether the yield varies from 30–50 %, whereas in dry ethyl alcohol higher yields, up to 60 % of the theoretical, have been obtained. The solubility of the hydrochloride is very slight in these solvents. Assuming that the precipitation of the hydrochloride is not interfered with by the other substances present—an admittedly unsound assumption—an attempt has been made to gain some idea of the proportion of spinacene present in the cod-liver oil fractions.

B.P. of fraction (3 mm.)	Iodine value	Weight taken g.	Solvent	Weight of hydrochloride	Yield assumed %	% spinacene in fraction
180°–200°	110	0·750	Dry ether	0·030	40	6·5
180°–200°	110	0·500	,,	0·019	40	6·2
200°–220°	129	1·056	Dry alcohol	0·080	55	9·4
					Average	7·7

These figures, approximations as they are, may give us a rough idea of the amount of spinacene in our fractions, and are supported by those obtained when in a similar fashion attempts were made to determine the yield of dodecabromide. The yield of insoluble bromide appears to depend on a number of factors, such as the rate of addition of bromine, temperature, the time of standing, and in particular upon the presence of traces of moisture. Wide variations in yield, from 22–45 % of the theoretical, have been obtained using pure spinacene in dry ether. The yields of insoluble bromide given on bromination of the fractions from cod-liver oil were as follows:

B.P. of fraction (3 mm.)	Weight g.	Weight insoluble bromide g.
180°–200°	0·5100	0·0310
,,	0·5110	0·0495
,,	0·1010	0·0107

Assuming an average yield of 35 %, these amounts of bromide correspond to the following percentages of spinacene, 5·3, 8·4, 9·2, average – 7·6 %.

Whilst the unsatisfactory nature of these calculations must be admitted, we are inclined to believe that the approximate agreement between the figures given by the two methods indicates that the amount of spinacene present may be taken as 8 %. If we accept this figure as an approximate estimate of the percentage of spinacene in our physiologically active fraction, we may proceed to make one or two other calculations in the hope that they will throw some light on the nature of the other unsaturated constituents:

Iodine value. The iodine value of spinacene is 371. The average for a large number of active fractions is 124. This indicates an iodine value of about 103 for the spinacene-free residue.

Acetyl value. A number of determinations on active fractions gave values of 208, 173, 174, 210, 217, 202, 204, averaging approximately 198. Corrected for the presence of 8 % of spinacene this becomes approximately 215.

Molecular weight. Direct determinations by the cryoscopic method gave values of about 300.

These approximations suggest that the other main constituent or constituents of the active fractions might be unsaturated alcohols containing one hydroxyl group and one ethylene linkage (*e.g.* $C_{18}H_{35}OH$, M.W. 270, acetyl value 207, iodine value 94; $C_{20}H_{39}OH$, M.W. 296, acetyl value 189, iodine value 91·3).

Unsaturated Alcohols in Fish-liver Oils.

The work of Tsujimoto and his colleagues in Japan has provided much valuable information regarding the substances present in the unsaponifiable fractions from a variety of fish-liver oils. Tsujimoto and Toyama [1922] describe two alcohols in the liver oil of *Hexanchus corinus.* One of these is a solid substance to which they give the formula $C_{20}H_{42}O_3$, and the name batyl alcohol, whilst the second is an unsaturated alcohol, selachyl alcohol, $C_{20}H_{40}O_3$, which on reduction is converted into batyl alcohol.

Evidence has been obtained that the solid alcohol isolated by us from cod-liver oil and the liver oil of *Spinax niger* is probably the same compound as the Japanese investigators have described under the name of batyl alcohol. Selachyl alcohol appears to form a large proportion of the unsaponifiable matter derived from the liver oils of certain fish, *e.g. Cirrhigaleus barbifer*, *Somniosus microcephalus*, *Chimaera* (sp.), *Lepidorhinus kinbei* and *Zameus* (*Scymnodon*) *squamulosus*.

Another unsaturated alcohol, believed to be oleyl alcohol, $C_{18}H_{36}O$, has been found by Toyama [1922] as the main constituent of the unsaponifiable matter from the liver oil of *Chamylodoselachus anguineus*. In view of these observations it was necessary to determine whether these alcohols occur in cod-liver oil, and if so, whether they are related to the physiological activity.

Before presenting the evidence we have accumulated on these questions it will be well to refer to another path along which enquiries were made. Surveying the other unsaturated alcohols of high molecular weight that have been isolated from natural sources it was obvious that attention should be paid to the alcohol phytol, $C_{20}H_{39}OH$, derived from chlorophyll. It appeared quite likely that small amounts of phytol might be absorbed by the animal organism during the digestion of green plants, and that the alcohol might appear as a constituent of the unsaponifiable fraction of their liver fats. The nature of the unsaturated alcohols present in our active fractions is also bound up with the nature of the product described recently by Takahashi, Nakamiya, Kawakami and Kitasato [1925] under the name "biosterin," and regarded by them as pure vitamin A. The most complete account of their work is given in a monograph, printed in English, but there have been several earlier papers in Japanese [Takahashi, 1922; Takahashi and Kawakami, 1923].

The following table gives the data regarding the alcohols that have been mentioned, together with the corresponding data for spinacene and "biosterin."

Table I[1].

	Selachyl alcohol	Oleyl alcohol	Phytol	"Biosterin"	Our active fractions allowing for 8 % spinacene	Spinacene
% C	73·0	80·8	80·0	81·0	77·7	87·8
% H	12·2	13·5	13·5	11·0	11·8	12·2
Mol. weight	328	270	296	402	300	410
Formula	$C_{20}H_{40}O_3$	$C_{18}H_{36}O$	$C_{20}H_{40}O$	$C_{27}H_{46}O_2$	$[C_{20}H_{36}O_2]$	$C_{30}H_{50}$
Iodine value	79·15	94	91·3	180	124	371
Acetyl ,,	272 (dihydric)	207	189	139	215	—
Refractive index	N_{20} 1·4691	N_{20}^{D} 1·4620	N_{20}^{D} 1·4640	N_{20}^{D} 1·52517	N_{20}^{D} 1·4705	N_{20}^{D} 1·4965
Boiling point	236°–239° (5 mm.)	207° (13 mm.)	202° (10 mm.) 145° (0·02 mm.)	147° (0·02 mm.)	180°–220° (1–2 mm.)	252° (5 mm.) 240° (2 mm.) 203° (0·15 mm.)
Density	d_{20} 0·918	d_4^0 0·862	d^0 0·852 d^0 0·864	d_{15}^0 0·9561	—	d_{15} 0·8587

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[1] The majority of the recorded values for oleyl alcohol, phytol, and spinacene have been checked during the course of this work on preparations made in our laboratory.

A superficial examination of the monograph published recently by the Japanese investigators might reasonably give the impression that they had isolated vitamin A in the pure state, but such a view is rapidly dispelled when the evidence they have presented is carefully examined, particularly in the light of a knowledge of the peculiar difficulties that are encountered in working with complex mixtures of fatty substances of high molecular weight, such as are represented by the unsaponifiable fractions of oils and fats.

Boiling point. The constancy of the boiling point of "biosterin" would at first sight appear to indicate its purity, but it has been our experience that although fractions boiling over narrow ranges can be obtained in distillations of such substances, these fractions are nevertheless often mixtures.

Thus, very active fractions have often been collected between 180–200° at 2–3 mm.—approximately the range at which "biosterin" would probably boil at the same pressure—but they have been found on examination to be highly impure. The difficulty of separating the components of these mixtures of substances boiling at temperatures close together is so generally recognised that it is difficult to understand how Takahashi and his colleagues can have imagined that they were obtaining a pure product after a single distillation from a modified form of retort. For our own part we recognise our failure to effect any appreciable separation of the constituents of the cholesterol-free material by repeated fractionation from Claisen flasks fitted with various types of fractionation columns, and suitably "lagged" to maintain control of the temperature difference between boiling and distilling liquid.

The difficulties encountered in these distillations were of the same type that we had previously encountered in an attempt to separate the esters of the fatty acids of butter by repeated fractionation [Channon, Drummond and Golding, 1924].

The description of "biosterin" as "a reddish yellow, transparent viscous, oily substance" is in our opinion clear evidence that the product must have been impure. Fractions obtained by us were usually coloured a pale lemon yellow unless the distillation had been unsatisfactory and bumping and splashing had occurred. The redistilled fractions were always crystal-clear, pale yellow oils, and only the material passing over at the higher temperatures was at all viscous. Incidentally, the alcohol phytol boils at the temperature given for "biosterin"—145–147° at 0·02–0·03 mm.—so that, whereas there may be little or no phytol in the unsaponifiable matter from cod-liver oil, there certainly must be considerable amounts present in a "biosterin" prepared from green leaves.

Analysis and molecular weight. "Biosterin" contains 81·0 % of carbon and 11·0 % of hydrogen, and is stated to have a molecular weight of about 400. It must be apparent that elementary analyses made on heavy oily materials of doubtful purity have very little value. Analyses of our own active fractions were made in order to gain information as to the amount of oxygen-containing substances (alcohols) present, but it would be unreasonable to attach any importance to the fact that many of these analyses can be made to fit formulae, *e.g.* C 77·7 %, H 11·8 %, and cryoscopic determinations giving values about 300 correspond very well with the formula $C_{20}H_{36}O_2$.

Unsaturation. "Biosterin" is reported to contain three double bonds corresponding to an iodine value of 190. The highest iodine value recorded for any of our active fractions is 140, and it is known that this fraction contained at least 8 % of spinacene which has an iodine value of 371.

The "biosterin" prepared from cod-liver oil was found to contain 80·87 % C and 11·13 % H, whilst that prepared from green leaves contained 79·88 % C and 11·88 % H. These products were of similar physiological activity although their iodine values were 180 and 125 respectively. This large difference in iodine values of products of similar physiological activity would seem significant. The description of the preparation of "biosterin" from plant tissues leaves no doubt that considerable amounts of phytol (iodine value 91) must have been present.

The yield of the bromide of "biosterin," prepared by the addition of bromine dissolved in glacial acetic acid to a solution of "biosterin" in ether, is stated to have been 85 %. This we are quite unable to confirm, for, as stated elsewhere, bromination of our fractions yielded only small amounts of the characteristic insoluble bromide of spinacene.

Refractive index and specific gravity. The refractive index of "biosterin" is given as 1·52517 and the specific gravity at 15° as 0·9561. Both values are much higher than any we have encountered during our work, or the values given by components such as spinacene, and we are inclined to regard these figures with suspicion.

Physiological activity. If we take as a measure of the physiological activity of the products the dose required to maintain good growth in a rat of 90–100 g. weight, it appears that the original cod-liver oil from which Takahashi obtained "biosterin" was represented by 1×10^{-3} g., whilst that for the majority of oils used by us was $5–6 \times 10^{-3}$ g. Our active fractions gave growth in daily doses of 5×10^{-5} g. whilst "biosterin" did so in a dosage of 1×10^{-5}–5×10^{-6} g. In other words, the degree to which the vitamin has been concentrated in "biosterin" and our active fractions is of the same order if regard be paid to the activity of the original oils, and yet we know that our preparations were highly impure. We are entirely at a loss to account for the observation of the Japanese investigators that harmful results follow the administration of an excess of the active substance. We have many times administered large amounts of highly active fractions without observing any ill effects, and the suspicion is aroused that Takahashi's material may have contained traces of a toxic impurity.

Action of "biosterin" on sensitised plates. The description of the experiments made by Takahashi and his colleagues on the influence of their active material on photographic plates leaves no doubt that they were observing the effect described by Russell [1908]. No "fogging" occurred when quartz or glass separated the plate from the "biosterin." These observations provide additional confirmation that materials possessing vitamin A activity do not emit a radiation capable of "fogging" a sensitised plate after passage through quartz, as was originally claimed by Kugelmass and McQuarrie [1924]. It was shown by Drummond and Webster [1925] that the experiments of the latter were faulty, and that the "fogging" was probably due either to the "Russell effect" or to the action of a phosphoresence which is emitted by silica after it has been exposed to ultra-violet light. Kugelmass and McQuarrie have recently admitted their mistakes [1925].

Attempt to determine the Nature of the Unsaturated Alcohols present in the Active Fraction from Cod-liver oil.

The iodine value and acetyl value, after correction for spinacene, suggest that the unsaturated alcohols contain one ethylene bond and one hydroxyl group capable of acetylation. The possibility of oleyl alcohol or phytol being present had to be investigated.

Both these alcohols yield fairly characteristic crystalline silver salts after conversion into the acid phthalic esters. Treatment of our active fractions with phthalic anhydride in boiling benzene solution appeared to bring about little or no combination, and only a few crystals of what was possibly a silver salt were obtained. The amount was too small for examination. Control experiments with samples of oleyl alcohol prepared from oleic ethyl ester by the method of Bouveault and Blanc [1904], and with phytol prepared from phaeophytin [Willstätter and Stoll, 1913] gave good yields (60–70 %) of the characteristic silver salts. It was also ascertained that the presence of spinacene does not interfere with the formation of these compounds. Takahashi has also recorded that his active fraction does not yield an acid phthalate by the usual method, but that combination occurs at the higher temperature of 120–130°. The description of his phthalate is, however, somewhat unsatisfactory. From this evidence and from a failure to obtain the phenylurethanes we concluded that neither phytol nor oleyl alcohol is present to any marked extent in our active fractions. Incidentally, it is of interest to mention that Javillier, Baude and Lévy-Lajeunesse [1925] showed by feeding experiments on rats that phytol does not possess the physiological action of vitamin A. Our preparations of phytol and oleyl alcohol failed to give a response to the colour tests for vitamin A, and feeding experiments also gave negative results. There remains the possibility that the selachyl alcohol described by Tsujimoto might be present, and that it might be responsible for vitamin action. As far as we are aware no derivative of selachyl alcohol has been described that is suitable for its detection, so that no means are available for ascertaining the first point. Indirect evidence that this substance is not vitamin A is given by the fact that the liver oil of *Scymnodon squamulosus*, of which the unsaponifiable matter consists almost entirely of spinacene and selachyl alcohol, has been found by us to be physiologically of very low activity.

The evidence presented above seems to rule out the possibility of vitamin A being identified with oleyl alcohol, phytol or selachyl alcohol. In our opinion the data available are quite inadequate to tell us whether or not the physiological activity is due to the presence of other unsaturated alcohols, for no separation of these in a state of purity has been achieved. No satisfactory answer to this question can be given until the active constituent has been isolated in pure condition or as a derivative suitable for purification. It was of interest to observe that during the course of exhaustive experiments to obtain solid derivatives suitable for purification and examination the vitamin activity of our fractions survived certain chemical treatments of the hydroxyl group of the alcohols present, *e.g.* acetylation, benzoylation and treatment with phthalic anhydride or substituted benzoyl chlorides, but in no case did it survive exposure to reagents that affected the unsaturated linkages, *e.g.* bromination, reduction.

Other Constituents of the Unsaponifiable Fraction.

It is obvious that the unsaponifiable fractions from oils such as cod-liver oil are highly complex mixtures which have as yet been but imperfectly examined. Amongst the components are the lipochrome pigments first detected by Salkowski [1863]. The yellow pigments of cod-liver oil remain unidentified, and neither the fresh oils nor the concentrated fractions present absorption spectra which it has been possible to measure. It has been found during the course of this work that there is a rough correlation between the physiological activity of the oils and the degree of pigmentation but the significance of this is uncertain. It is also uncertain whether the pigments are volatile during distillation.

The association of vitamin A with the lipochrome pigments has been observed many times [Steenbock, 1919; Steenbock and Boutwell, 1920; Rosenheim and Drummond, 1920], but a number of exceptions having been described [Palmer and Kempster, 1919, 1, 2, 3; Drummond and Coward, 1920], it has been generally concluded that it is of no significance as regards the identity of the former substance. Drummond [1919] observed that a pure specimen of recrystallised carotene possessed no growth-promoting powers, whereas Steenbock has once reported that a specimen of carotene induced growth [Steenbock *et al.*, 1921]. No confirmation of this statement has appeared from their laboratory as far as we are aware.

More recent studies that we have made on a specimen of carotene recrystallised four times, M.P. 167·5°, suggest that the lipochrome possesses no growth-promoting activity such as vitamin A shows, but that it may, unless carefully purified, be contaminated by traces of the latter substance. This, at least, appears to be the most satisfactory explanation of the long survival of the animals in some tests when compared with the rapid failure of those on the basal ration. Lycopin, prepared from tomatoes, is likewise inactive. We are convinced, therefore, that neither carotene nor lycopin is vitamin A.

Xanthophyll has not yet been prepared by us in sufficiently pure condition for a satisfactory test.

SUMMARY.

1. Further confirmation has been obtained of the concentration without loss of vitamin A in the unsaponifiable fraction of cod-liver oil, provided the preparation is carried through with precautions against oxidation.

2. The concentrate contains no detectable traces of iodine or nitrogen, so that these elements do not appear to be related to the physiological action of the oil in promoting growth.

3. Approximately 50 % of the unsaponifiable matter from cod-liver oil is cholesterol, which may be removed quantitatively without loss of vitamin activity.

4. Vitamin A is volatile in superheated steam.

5. Distillation of the cholesterol-free residue in a high vacuum did not result in a satisfactory separation of the components. Vitamin A passes mainly between 180–220° at 2–3 mm.

6. Chemical examination of active distillates indicated the presence of (*a*) a saturated solid alcohol (M.P. ca. 60°), (*b*) the unsaturated hydrocarbon spinacene, (*c*) one or more than one unsaturated alcohol, boiling about 200° at 2–3 mm.

7. Spinacene and the solid alcohol are without vitamin A action.

8. It has not been possible to decide whether vitamin A is identifiable with one of the unsaturated alcohols.

9. The unsaturated alcohols, oleyl alcohol ($C_{18}H_{36}O$) and selachyl alcohol ($C_{20}H_{40}O_3$), which have been described as constituents of other liver oils, are not identifiable with vitamin A. The unsaturated alcohol, phytol ($C_{20}H_{40}O$), which is present in the chlorophyll molecule, is also devoid of activity.

10. A criticism is made of the claims of Takahashi to have isolated vitamin A and to have identified it as an unsaturated alcohol $C_{27}H_{46}O_2$ (" biosterin ").

11. Brief reference is made to the possible relation between the lipochrome pigment of cod-liver oil and the vitamin activity of the latter.

We wish to express our sense of indebtedness to the Medical Research Council for having so generously provided large grants to cover the cost of the research over a number of years.

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CLII. ON THE TECHNIQUE OF TESTING FOR THE PRESENCE OF VITAMIN A.

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The technique that has for some time past been employed in this laboratory for testing for the presence of vitamin A in foodstuffs, or fractions isolated during our efforts to determine the nature of that substance, has been described in previous publications [Drummond and Coward, 1920; Drummond and Watson, 1922, 1]. Until quite recently we placed considerable trust in this method, which, in broad detail, consisted of feeding young rats on an artificial ration purified as far as possible from fat-soluble vitamins until the deprivation resulted in a cessation of growth. The response in growth that followed the administration of the foodstuff, or other substance, was then interpreted as an indication of the amount of vitamin A that had been supplied. Our belief that this test, standardised as carefully as possible, yielded trustworthy results, was not disturbed when it was clearly demonstrated, first by McCollum and his co-workers, that the vitamin A is a substance distinct from the antirachitic factor, now somewhat generally referred to as vitamin D, because there was then no evidence to show that the latter substance might interfere with the test.

Steenbock and his colleagues have, however, by a very careful series of researches demonstrated that the cessation of growth that is exhibited by animals maintained on the more or less standard type of basal diet employed in testing for vitamin A may not always be due to a deficiency of that substance, but that in some cases it may be caused by an inadequate supply of vitamin D.

Recognising the importance of their observations, Steenbock, Nelson and Black [1924] devised a modification of the technique usually employed in testing for vitamin A, which consisted of supplying the antirachitic factor throughout the experiment either by a supplement of aerated cod-liver oil—it having been shown by McCollum, Simmonds, Becker and Shipley [1922] that the vitamin A in that oil is destroyed by 12–28 hours' oxidation by a current of air at 100°, whereas its antirachitic action survives—or by regular exposure of the test animals to radiations, from a suitable source, such as a quartz mercury-vapour lamp, which are now known to endow certain substances with antirachitic power. On the whole, they prefer the latter alternative, and propose to maintain the animals on their ordinary basal diet of purified caseinogen, agar, yeast, salt mixture and dextrinised starch, but to guard against the incidence of disturbances arising from a shortage of the antirachitic substance by exposing the animals to the radiations of a quartz mercury-vapour lamp for a short period, say 10 mins. every day. In their experiments the control rats receiving no irradiation showed some growth, with incidence of ophthalmia in from four to six weeks. Those which received the same food mixture, but in addition were exposed to the radiations every day, grew considerably more, but showed signs of ophthalmia at about the same time as the controls. The present paper reports results which in essentials confirm those to which we have just referred, and we record them, in the first place, because they were arrived at from a somewhat different line of approach to the problem, and in the second, because we believe that our modification of the usual technique is slightly superior to that proposed by the investigators we have mentioned.

We were led to this subject by an investigation, recently reported by Drummond, Rosenheim and Coward [1925], during which it was found that cholesterol of a fairly high degree of purity[1] after exposure to the radiations of a quartz mercury-vapour lamp promotes a resumption of growth when administered to rats which have ceased to grow on the standard diet deficient in vitamin A.

The fact that cholesterol treated in this manner is also endowed with marked potency as an antirachitic has now been demonstrated with certainty [Hess, Weinstock and Helman, 1925, 1, 2; Hess and Weinstock, 1925; Steenbock and Black, 1925; Rosenheim and Webster, 1925].

At first we were inclined to believe, from the very prompt recovery which our animals showed in growth, that the radiation had also effected either a synthesis of vitamin A, or of some substance possessing similar physiological action. On the other hand, doubts were raised by two facts. In the first place the irradiated cholesterol, although it gave colour reactions differing markedly from those of the untreated compound[2], failed to give the colour tests that have been studied in this laboratory [Drummond and Watson,

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[1] The purity of samples of cholesterol is an important matter in work of this type, where a minute trace of impurity may well be the precursor of the active substances formed during exposure to short wave-length radiations. Dr O. Rosenheim has given us the benefit of his wide experience of cholesterol and related compounds, and has emphasised the necessity of at least two saponifications with repeated crystallisations from more than one solvent before anything approaching pure cholesterol can be obtained. The product employed by us was three times subjected to hydrolysis with boiling alcoholic potash, with many intermediate and subsequent crystallisations, totalling 27 in all, alcohol, ether and light petroleum being used as solvents. It melted at 148·8° (uncorr.). Melting points as high as 150° have been recorded, and it is doubtful whether a product melting much below 149° can be regarded as satisfactory.

[2] The colour reactions of irradiated cholesterol, and their significance, will be the subject of a later communication by Dr O. Rosenheim.

1922, 2; Rosenheim and Drummond, 1925], which we are strongly inclined to believe are given by the substance known as vitamin A. Secondly, the response in growth that in most cases followed the administration of irradiated cholesterol was irregular, and could not be correlated in a quantitative manner with the amount of the supplement.

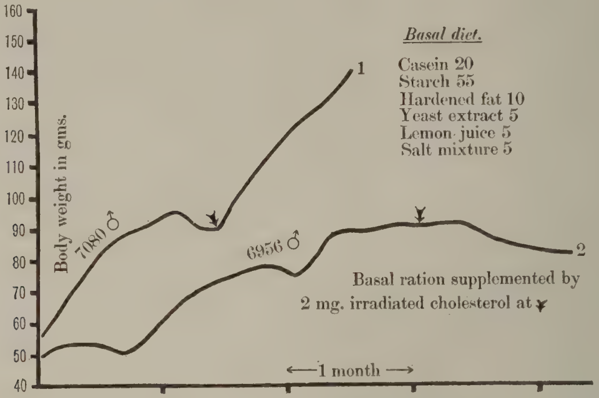

Fig. 1. Typical curves illustrating the two extreme types of response to an administration of 2 mg. of irradiated cholesterol daily. Curve 1 shows the prompt resumption of growth usually shown by animals which have previously shown the characteristic failure of growth at 4–5 weeks on the basal ration. Curve 2 shows failure of the irradiated cholesterol to cause resumption of growth in an animal that has been maintained on the deficient basal ration for 3 months.

The significant irregularities encountered were of two types. In some cases, illustrated by curve 2 in Fig. 1, the addition of the irradiated cholesterol produced no resumption of growth, but occasionally appeared to postpone the final decline. An analysis of the cases in this group revealed the interesting fact that practically all the animals that failed to respond had been maintained on the basal ration for a relatively long time; in the case illustrated by curve 2 (Fig. 1) the rat had been on the basal diet for three months before receiving the supplement of irradiated cholesterol.

The second type of irregularity that aroused our suspicion was shown by animals such as those whose growth curves are illustrated in Fig. 2. In these cases the resumption of growth on giving the irradiated cholesterol was prompt, and the rate sometimes almost normal, but after about four weeks a retardation set in followed by a rather rapid fall in weight. The important point, to our minds, was that the recovery we have described did not appear to be correlated with the dose of irradiated cholesterol given. This was indicated by the fact that daily supplements of 1–10 mg. appeared equally efficient in causing recovery, but, more particularly, by the fact that increasing many-fold the amount of the supplement after the second cessation (Y) of weight had occurred did not in a single case produce a further stimulation of growth (curve 4, Fig. 2). If, however, a small amount of cod-liver oil, or of a fraction rich in vitamin A prepared from cod-liver oil, was administered at that point, a prompt and steady increase of weight again occurred (curve 5, Fig. 2). From this it may reasonably be concluded that the irradiated sterol was not supplying the substance known as vitamin A, and that the temporary development that followed the administration of this substance must have been due to another cause. To attempt an explanation of those results is, perhaps, unwise, until much more has been achieved in the experimental field, but provisionally the hypothesis tentatively advanced by Steenbock may be accepted, since it is not at variance with any of the observations we have recorded. The idea that the initial cessation of growth may be brought about by a deficiency of vitamin D in the basal diet at a time when the animal has not expended the reserves of vitamin A in its own tissues is one that fits in not only with the temporary response in growth that may follow the administration of the antirachitic vitamin D (irradiated cholesterol), but also with the fact frequently observed by us, that after a long period of maintenance on the basal ration animals usually fail to show any response to this substance; the long period of deprivation having, presumably, completely exhausted their reserves of vitamin A (curve 2, Fig. 1).

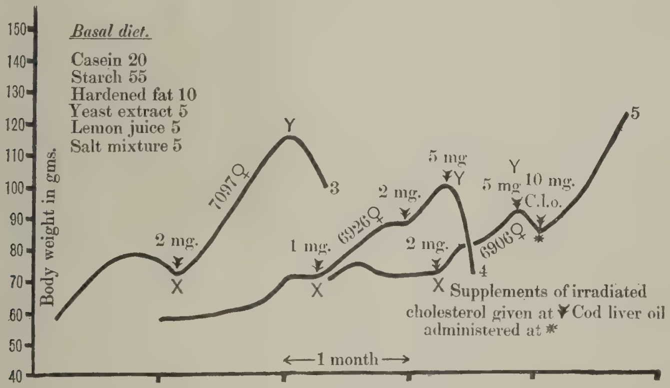

Fig. 2. Typical curves illustrating (3) rather rapid cessation of growth and decline (*Y*) after temporary resumption of growth on irradiated cholesterol (*X*). Curve 4 illustrates failure of growth in spite of increasing dose of irradiated cholesterol. Curve 5 illustrates power of cod-liver oil to prevent this decline.

There can be no question that Steenbock is right in emphasising the very complicated nature of the inter-relationships that exist between the many factors that determine growth in the young animal. Viewed from the standpoint of the value of tests for vitamin A the results of these observations are of considerable importance. In the past we have regarded the power to induce steady growth for a period of not less than three or four weeks as evidence that the substance, added as a supplement to the basal ration, contained the vitamin A. Judged by this criterion we should conclude that the irradiation of cholesterol by ultra-violet radiations synthesises a substance identical with, or possessing similar physiological action to vitamin A.

Obviously, however, unless the animals are protected throughout the test from a deficiency of the antirachitic substance, the method will be open to grave error. Indeed, we fear that no small proportion of the results that have been published on the occurrence and properties of vitamin A may have to be revised by tests in which this precaution has been taken.

We have referred to the means suggested by Steenbock for modifying the animal test for vitamin A. We have found it more convenient to employ irradiated cholesterol as a source of vitamin D in the basal diet. We usually prepare the "activated" material by radiation of either thin films of carefully purified cholesterol spread over the bottom of shallow flat dishes, exposed to air, or a thin film spread on the inner surface of a spherical quartz flask, evacuated by a mercury pump, and slowly rotated mechanically so as to ensure equal exposure of the whole surface. We are inclined to believe that the second method is preferable. In both cases we employ a Cooper-Hewitt quartz mercury-vapour lamp (220 v., 7·5 amp.) at a distance of 25 cm. for 2 hours, as a source of ultra-violet rays[1].

The animals are fed on a basal ration similar to that used by us for some years past, but from which we now exclude all fat. The mixture is compounded as follows:

Purified caseinogen	15 parts
Pure rice starch	70 ,,
Yeast extract	5 ,,
Salt mixture	5 ,,
Lemon juice	5 ,,

In a large number of experiments we have found that the exclusion of fat does not apparently disturb the normal behaviour of the animals on the basal ration[2]. As this is so, and as we have suspected that slight differences in the nature of the fats employed have at times been responsible for abnormal results, we decided to exclude them as being unnecessary potential sources of error.

The basal diet given above is, however, supplemented from the beginning of the experiment with 1·0 mg. of irradiated cholesterol daily, this being administered to the animals separately in the form of one drop of a solution in liquid paraffin. It may also be incorporated in the diet to the extent of 20 mg. %, so that each rat obtains 2–3 mg. daily.

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[1] We are indebted to Prof. E. C. C. Baly, F.R.S., for much advice in connection with the use of these lamps. Too frequently it is not borne in mind that they exhibit "decay," that is that the proportion of radiations of short wave-lengths falls off with use. The "decay" would appear to be due to changes in the transparency of the silica walls of the lamp, by which they become more opaque to the shorter rays. This occurs more readily if the lamp is run "cold" at a low voltage, and, apparently, within limits, the hotter the lamp becomes when working the longer will it retain its transparency to the physiologically active rays. A rough test, that saves the trouble of periodic examinations of the light by optical methods, is the formation of ozone. As long as the smell of this gas is marked it is certain that the lamp is emitting rays that are in the part of the spectrum in which occur those responsible for the antirachitic effect.

[2] The successful rearing of rats for several generations on diets devoid of fats has already been reported [Drummond and Coward, 1921].

Typical growth curves of animals during this preliminary period are illustrated in Fig. 3 from which it will be seen that the initial growth is considerably greater than is shown by similar groups not receiving the irradiated cholesterol. Instead of the characteristic retardation of growth appearing at about the fourth week, when the animal weighs about 90 g., the supplemented basal ration enables the animals to grow for 5 to 6 weeks, reaching a weight of about 110–130 g. before their growth stops. Further maintenance on the basal diet, although enriched by the antirachitic factor, results in decline and death, and, as far as we have been able to ascertain, this end can only be prevented, and growth re-established, if a supply of the vitamin A is promptly administered[1].

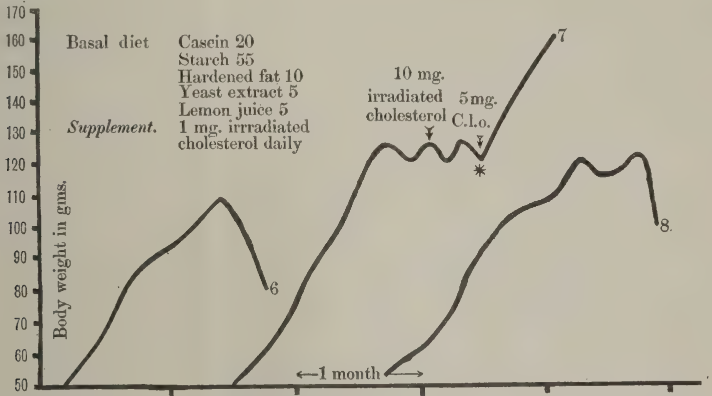

Fig. 3. Curves 6 and 8 show typical behaviour of rats fed on basal diet containing no fat and supplemented with 1 mg. irradiated cholesterol daily. The onset of the decline is sharp. Curve 7 shows failure of extra irradiated cholesterol to induce further growth, but prompt recovery on adding 5 mg. cod-liver oil.

It is interesting to note that these curves are rather sharply "peaked." The decline sets in almost as soon as the rats cease growing, and there is seldom a period of maintained body weight as there usually is on the old basal diet (Fig. 1).

It is unfortunate that the modification of the technique which we have described increases the time required to carry through a test to detect the presence of vitamin A in a substance.

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[1] The administration of substances, in particular those relatively rich in vitamin A, to animals during these tests presents some difficulty. It is useless, in our opinion, to incorporate them with the diet. Zilva and Miura [1921, 1, 2] introduced the use of olive oil aerated for many hours at 100° to destroy vitamin A, as a diluent, and administered measured drops of a solution in this medium of the substance to be tested. Even this procedure, which was adopted by us for some time, appears to be open to criticism, because the aeration of the olive oil results in the formation of substances, loosely termed peroxides, which appear to have a destructive action on the vitamin A present in the substance to be tested [Fridericia, 1925].

We have more recently employed liquid paraffin (medicinal) as a diluent. This has the advantage of being free from vitamin A, and of being an inert solvent suitable for use with fatty substances. On the other hand we are not yet satisfied that absorption of substances dissolved in this medium is quantitative.

It is for this reason that we are concentrating our attention on methods which will replace the expensive and time-consuming feeding experiments with animals. In a recent communication [Rosenheim and Drummond, 1925] we have outlined colour reactions which we believe are given by vitamin A, and which we hope will before long be made the basis of methods of quantitative estimation. We have in the last month or two attempted to compare the sensitivity of these colour reactions with that of the rat-feeding tests. It has proved a harder task than we anticipated, and we do not wish to commit ourselves to a definite opinion until further work has been completed. It may be said, however, that the sensitivity of the colour reactions with trichloroacetic acid or dimethyl sulphate, as determined by their value in comparing the vitamin A content of different samples of cod-liver oil, or different dilutions of the same sample of cod-liver oil in a neutral solvent, seems to be of about the same order as the animal test. The reaction with arsenic trichloride is decidedly more delicate.

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CLIII. MAMMARY SECRETION. VI. VITAMIN B AND THE LACTATING RAT'S DIET.

1. THE QUANTITATIVE RELATION OF VITAMIN B TO PROTEIN (*continued*).

2. VITAMIN B REQUIREMENT OF THE LACTATING AND NON-LACTATING RAT. (PRELIMINARY NOTE.)

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(*Received October 7th, 1925.*)

1. THE QUANTITATIVE RELATION OF VITAMIN B TO PROTEIN (*continued*).

INTRODUCTION.

At the present time there appears to be no definite knowledge as to the minimum amount of any vitamin required for growth and the maintenance of health. Quantitative work is, as yet, very much in its infancy (especially in respect of vitamin B); probably our technique is not sufficiently good to permit of any very accurate work in this field of research, and such work as there is lies along other lines. For example, the distribution of the various vitamins in different foods has been the subject of much research, whilst very few observers have attempted to ascertain the animals' actual needs in respect of these elusive factors (except the antiscorbutic requirements of the guinea-pig and monkey). Many dietetic workers (using rats) give as much as 5·0 g. marmite for every 100 g. (approximately) of synthetic food. It is probable that this figure is adopted because it is the amount used in the diet laid down by the Accessory Food Factors Committee when testing for the presence of vitamin A [Medical Research Council, 1924]. It obviously supplies the needs of the rat, but does it not also provide excess of the vitamin? For example, a young rat (about 60 g.) bred in this laboratory and fed on a synthetic diet from weaning (21 days of age) would eat approximately 0·6 g. marmite per day, whilst Osborne and Mendel [1922] find that a daily ration of 200 mg. of their yeast is enough to provide for the rat to attain "the average adult size within the average normal time." One cannot assume that the English yeast extract is absolutely equivalent to the American yeast, but allowing for some variation there is a big difference between 0·6 g.

and 0·2 g. From this it appears that the 5·0 g. marmite to 100 g. synthetic diet may provide an excess of vitamin B. There is so far no really good method for estimating vitamins, and therefore even the synthetic diet which is spoken of as "vitamin-free" may be only relatively free, for there is no proof that the constituents of that diet are *absolutely* vitamin-free. The only fact proved by experiments is that such a diet does not contain sufficient of any vitamin to support life, and hence all vitamin experiments can only be regarded as comparative.

In a previous communication [Hartwell, 1924, 2] it was shown that a definite quantitative relation exists between the amount of protein and the need for vitamin B in a lactating rat's diet. In this work, bread, food casein, and tomato juice were used, and it was thought advisable to make more experiments along similar lines using synthetic diets in which the protein content could be more accurately estimated. With this object in view the experiments to be described in this paper were carried out.

EXPERIMENTAL DATA.

During gestation the mother rats received a good mixed diet of kitchen scraps, supplemented by bread and milk. The synthetic diet was started as soon as possible after the birth of the litter (never more than 18 hours) and continued for 21 days, at which time the young were weaned. In one or two experiments, when the diet was low in protein (10 %), the baby rats were small and weakly, and would not eat for themselves at the usual time (16th–18th day). In such cases the young were left longer with the mother and the diet was continued for three days after the young were seen to be eating from the mother's food.

The general method of experiment (daily weighings of mother and babies, etc.) has been fully explained in previous papers [Hartwell, 1921, 1; 1924, 2].

Foods. The dry constituents (caseinogen, potato starch and salt mixture) were mixed in bulk; the butter and marmite, with water, were added daily to weighed amounts of the dry mixture. Three different proportions of caseinogen were used, as follows:

	Diet A g.	Diet B g.	Diet C g.
Caseinogen	20	10	30
Potato starch	64	74	54
Salt mixture	4	4	4
Washed butter [Hartwell, 1924, 1]	12	12	12

Vitamin B. Marmite was used as a source of this factor, the same sample being used for all the experiments. The marmite was accurately weighed in a beaker, dissolved in warm water, and by this means transferred to a graduated flask. When cold the volume was made up to the mark. The marmite solution was made daily in hot weather, and every other day when it was cold; the required amount was pipetted out and mixed evenly with the food.

Experiments.

1. *Diet A* (20 % caseinogen). A series of experiments was made in which decreasing strengths of marmite were used, viz. 20, 15, 10, 5 %, and no marmite at all. The mothers and young were watched carefully for any abnormal symptoms.

The results are given in the following table:

Number of exps.	G. of marmite per 100 g. diet	*Condition of young rats*	*Condition of mother rats*
2	8	Normal	Very slight loss in weight, otherwise normal
9	6	8 litters normal; 1 litter slight spasms	Slight loss in weight, otherwise normal
4	4	All 4 litters had spasms, and 3 litters screaming fits. 9 out of 24 died. Fur in good condition, but no young normal	Mothers lost weight, but appeared to be well
3	3	All 3 litters spasms and screaming fits; whole litter died	,, ,,
7	2	Condition very poor; 4 whole litters died. Babies which survived were weakly for at least 10 days after weaning	Great loss in weight (in the worst case a rat which weighed 286 g. at birth of litter weighed only 173 g. at weaning)
7	0	Condition of young very poor indeed; 26 out of 40 died, and the survivors were not normal for some time after weaning	Great loss in weight (the worst case being a loss of from 306 g. to 210 g.)

Thus it appears that with diet A (20 % caseinogen) 6 g. of marmite to 100 g. diet in the mother's food is generally enough to protect the young from bad effects, and 8 g. is a complete safeguard.

2. *Diet B* (10 % caseinogen). Three series of experiments were made, using 15, 5 and 2·5 % marmite solutions respectively. This diet was by no means satisfactory; the mothers lost weight badly and the young were of small size, and some were weakly. In spite of these drawbacks, no spasms or abnormal symptoms of any kind were seen in the suckling rats when the mothers were receiving only 5 % marmite, and with 2·5 % no spasms occurred, but some of the young showed slight incoordination of movement.

3. *Diet C* (30 % caseinogen). One series of experiments only was made and the strength of marmite used was 15 %. All three litters were abnormal and exhibited typical spasms and screaming fits, so it is obvious that with a higher percentage of protein, the 15 % marmite is no longer sufficient to afford protection to the young.

4. *Test of diet A for freedom from vitamin B.* This synthetic diet was expected to be free from vitamin B, but it was thought advisable to make a test on animals from the same stock as were being used for the other experiments. Accordingly 12 young rats (6 of each sex) were fed on diet A + water; the results were quite in agreement with those of other workers using a vitamin B-free diet.

RESULTS AND COMMENTS.

The experiments described in this paper afford evidence that there is a definite relation between the protein content and the vitamin B of the lactating rat's diet. Thus 15 % marmite afforded complete protection to the young when the mother's diet contained 10 % of protein, almost complete protection when the mother rat was eating 20 % protein, but was quite inadequate for the higher protein intake (30 %). With a diet containing 20 % of protein, 20 % marmite afforded complete protection, and 15 % was almost enough, but with lower percentages of marmite the young became correspondingly worse (see Fig. 1) until with 5 % many died.

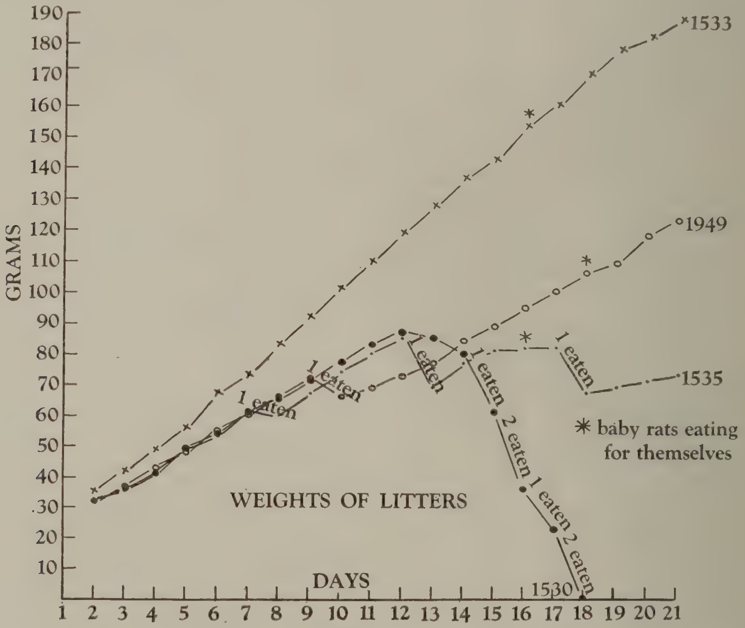

Fig. 1.

Mothers' diets.

× —— ×	1533	25 g. diet A	+	10 cc. of	15 %	marmite	solution
○ —— ○	1949	,,	+	,,	10 %	,,	,,
• —— •	1535	,,	+	,,	5 %	,,	,,
● —— ●	1530	,,					

It is rather extraordinary that there appeared little difference in the condition of the young when the mother received 5 % or no marmite, especially since the 5 % level of intake appears to meet the needs of the young growing rat in respect of vitamin B (see second part of paper). A possible explanation may be that the 5 % is so little compared with what she really needs that it makes only a slight difference if she receives none. It is also remarkable that the rat can rear any young at all on a diet free from vitamin B. Presumably she does so at great expense to herself, for it is a well-known fact that the mother safeguards her young by sacrificing her own tissues, as shown by great loss of weight. The young of mothers fed on a diet lacking in vitamin B are by no means normal, but 14 out of 40 survived weaning and eventually became healthy rats, though they were by no means fit for about two weeks after weaning (Fig. 2).

Another point which is well illustrated in the experiments described here is that a considerable loss in weight of the mother rat frequently results in the survival of her offspring, while another mother on the same diet maintains her weight (or loses slightly) and her offspring all die (Fig. 2). This point has been alluded to in previous papers [Hartwell, 1921, 2, and Hartwell, 1924, 2].

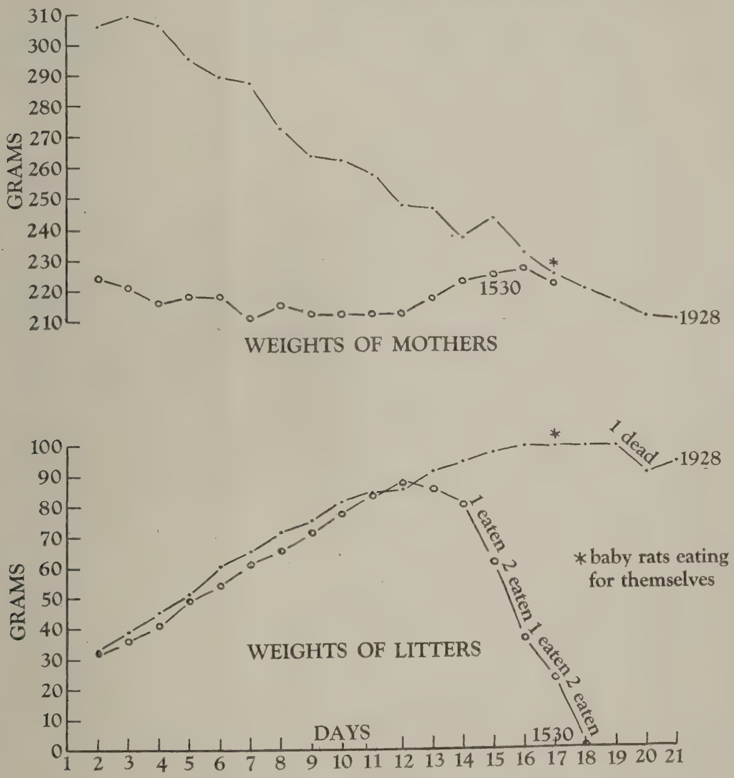

Fig. 2.

Mothers' diet.

• —— • 1928 } Diet A and no marmite, i.e. a synthetic diet containing no vitamin B.
○ —— ○ 1530 }

Diet B (10 % protein) gave very poor results.

The mothers lost weight, and the young were undersized and weakly. It is probable that the low percentage of protein is responsible for these bad effects. With 15 % marmite the young were still of low weight, though in rather better condition. It may be that the yeast protein is responsible for the improved condition. The mothers, on an average, ate 25 g. food, *i.e.*

2·5 g. caseinogen per day. With this they would receive 1·5 g. marmite, so it is conceivable that the protein ration of the synthetic diet was supplemented by the yeast protein.

In spite of the young being weakly, no spasms were observed even with as little as 2·5 % marmite, and since it is quite possible that weakly babies might be more susceptible to any deficiency in the mother's milk, this may be an added proof of the proportional relation of vitamin B to protein in the lactating rat's diet. The addition of large quantities of vitamin B to this low protein diet were ineffective in saving the mothers' weights to any great extent. This is shown in the following table:

Strength of marmite %	Average loss in weight of mother rat g.
15	61
5	70
2·5	80

2. VITAMIN B REQUIREMENT OF THE LACTATING AND NON-LACTATING RAT (PRELIMINARY NOTE.)

In a previous communication [Hartwell, 1924, 3] it was shown that, with diets rich in protein, the lactating rat must have a greater supply of vitamin B than at other periods of her existence, otherwise she is unable to rear her young. As a result of this work, the problem arose as to how much more vitamin B is required by the lactating than non-lactating rat.

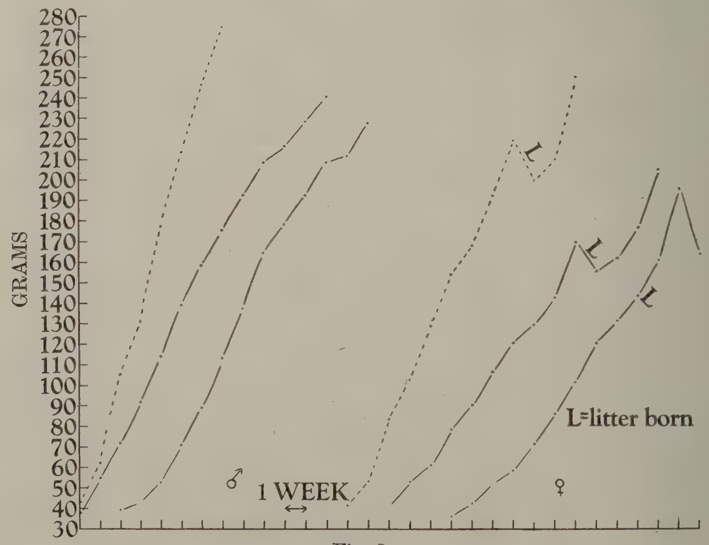

Fig. 3.

- - - - Control rats—diet of kitchen scraps and bread and milk

• —— • Rats fed on diet A + 5 % marmite (25 g. diet A to 10 cc. of 5 % marmite solution)

Twelve young rats were fed on a diet consisting of 20 g. caseinogen, 64 g. potato starch, 4 g. salt mixture, 12 g. butter (washed), 2 g. marmite (which contain 27·35 % water)[1]. At the end of 6 months they were fit and healthy. Some of their growth curves are given as examples (Fig. 3). The rate of growth is better than that described by Donaldson [1924] as the normal curve for the rat.

It was found with this diet that the mother rat required 6–8 g. marmite to enable her to rear her young successfully.

This work proves that on an average diet (such as 20 % protein, etc.) the lactating rat needs at least 3–4 times as much vitamin B as does the non-lactating animal, otherwise she cannot rear her young. These results are in agreement with the work of Grant [1924] who found that a similar ratio existed between the needs of the lactating and non-lactating rat.

SUMMARY.

1. There is a quantitative relation between the protein and vitamin B in the lactating rat's diet. On a synthetic diet containing 20 % caseinogen, 6–8 g. marmite per 20 g. caseinogen are needed for the mother rat to rear her young; with 4 g. marmite the babies exhibit spasms and screaming fits, while with 2 g. the young are in a still worse condition and the majority of them die.

2. On a synthetic diet containing 20 % caseinogen the lactating rat requires at least four times as much vitamin B as does the young growing animal, otherwise she cannot rear her young successfully.

3. The lactating rat can rear some of her young on a synthetic diet free from vitamin B, but she loses weight during the lactation period. Her young are weakly when weaned, but eventually recover.

4. On a synthetic diet containing 10 % of caseinogen the lactating rat loses weight and her young are under-sized.

The expenses of this research were defrayed by a grant from the Medical Research Council.

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[1] I am indebted to the Marmite Food Extract Co. for supplying me with this figure.

CLIV. A COLOUR REACTION FOR DISULPHIDES.

By ERNEST WALKER.

From the Department of Biochemistry, Oxford.

(*Received October 19th, 1925.*)

The colour reaction here to be described was discovered in the course of some work upon the sulphur constituents of tissue. Reference to the literature, however, revealed that the reaction had been applied previously in the specific case of cystine. As the reaction appears to be capable of wide application and is simple to carry out, it is proposed to give some account of it here and to mention a few of its applications.

The actual test is simply a modification of the well-known nitroprusside reaction for the sulphydryl group, a solution of potassium (or sodium) cyanide being used instead of ammonia or other alkali. In the case of a solution containing a disulphide, a few drops of 5 % aqueous sodium nitroprusside are added, followed by 3–5 drops of 10 % aqueous potassium cyanide. The final reaction of the solution must be alkaline. If the disulphide is present in high concentration, the colour, a deep magenta, develops immediately; if present in low concentration, there is a delay of a few minutes. For example, an alkaline solution of cystine, containing 1 part in 1000, shows a slight delay and the colour may not reach its maximum intensity for 20 minutes. A solution containing 1 part in 10,000 shows a somewhat longer delay and the final coloration is only a faint pink. This concentration (1 : 10,000) about represents the limit of sensitivity of the reaction. In the examination of tissues the test may be performed in the presence of a high concentration of ammonium sulphate, as recommended by Hopkins for the sulphydryl test. In all cases the colour remains stable for an hour or longer. It is important to note that the reagents used will give a similar colour with the —SH group, if such be present; this may be controlled by first testing with sodium nitroprusside and ammonia. Furthermore, the slight delay which occurs in the disulphide reaction serves to differentiate it from the instantaneous colour given by sulphydryl compounds.

With regard to the mechanism of the reaction, there can be little doubt that the cyanide effects the reduction of the disulphide grouping to the sulphydryl grouping and that the latter then gives the normal nitroprusside reaction. Mauthner [1912] showed that cystine is reduced to cysteine by aqueous potassium cyanide, and more recently Abderhalden [1923] has made use of this reducing power of cyanide in investigating the oxidation of cysteine.

Sullivan [1925], in describing a specific colour test for cysteine, states that this colour reaction is given by cystine in the presence of sodium cyanide, owing to the reduction of the cystine to cysteine by the cyanide.

The following is a brief description of a number of miscellaneous applications of the disulphide colour reaction.

In the first place, all disulphides tested have given a positive reaction. These are oxidised glutathione, cystine, dithiodiglycol[1], dithiodiglycollic acid and diethyl disulphide. With regard to the application of the test to tissues, it has been possible to demonstrate the presence of a disulphide grouping (presumably due to cystine) in hair, nails and the horny layer of skin. The hair used was that from a piece of white calf-skin and it was necessary to soak it in dilute acid in order to obtain a distinct reaction. The test has also been applied successfully to tissue (skin and water-extracted muscle) in which the sulphydryl group had previously been oxidised by hydrogen peroxide.

One case in which the colour reaction proved of use is described in the paper which follows this.

Applied to normal urine the test usually gives a faint positive reaction, corresponding to a disulphide concentration of 1 in 10,000. At such a dilution no quantitative significance is attributed to the test.

Harris [1923] has shown that native ovalbumin gives no sulphydryl reaction, but that on denaturation it gives a vivid reaction. He suggests that the precursor of the —SH formed on denaturation is not a —S—S grouping. The application of the disulphide reaction to native ovalbumin gives a negative result, thus substantiating Harris's suggestion.

Blood serum gives no —SH reaction, either in the native state or after coagulation. In the native state, serum gives only a very faint disulphide reaction, such as is given by a disulphide concentration of 1 in 10,000, although some two-thirds of its total sulphur is supposed to exist in the form of cystine. On the other hand, serum gives a vivid disulphide reaction after coagulation by heat. This change is thus analogous to that which occurs in ovalbumin, reading —S—S— for —SH, and is in agreement with the recent observation of Hopkins [1925] that thiol compounds react with serum proteins only after denaturation. What the precursor of the disulphide group is, it is difficult to say, but the facts suggest that the disposition of the cystine moiety in serum albumin is not simple.

SUMMARY.

A colour reaction for the detection of the disulphide grouping is described. The reagents used are potassium cyanide and sodium nitroprusside.

A few illustrations of the applicability of the colour test are briefly described.

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[1] It was observed that this compound is readily reduced, both aerobically and anaerobically, by water-extracted muscle powder. Being neutral in reaction it should prove more serviceable than thioglycollic acid in the investigation of biological oxidations.

Harris's suggestion, that the precursor of the sulphydryl group formed on denaturation of ovalbumin is not a disulphide linkage, is confirmed. It is also shown that blood serum in the native state gives no disulphide reaction, but gives a vivid reaction after coagulation.

I wish to record my thanks to Professor Peters for his interest and advice.

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CLV. THE SULPHYDRYL REACTION OF SKIN.

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(*Received October 19th, 1925.*)

In a preliminary communication [Walker, 1924], describing a nitroprusside reaction given by skin, it was shown that this reaction is definitely located in the metabolically-active layers of the skin. At that time it was not realised that the nitroprusside reaction of skin had already been described by Buffa [1904], in a paper in which he deals with the colour reaction given by many tissues on treatment with nitroprusside and sodium hydroxide. There can be little doubt that the reaction is due to the presence of the sulphydryl grouping, and the view was advanced in the preliminary communication that the substance responsible for the reaction was closely allied to the thermostable sulphydryl constituent of muscle, but was not identical with it. The only reason for considering the two constituents as not identical was based upon a slight difference in their respective reducing powers. Since then it has become apparent that this difference is simply one of degree and hence there is now no reason to suppose that different substances are responsible for the nitroprusside reaction of skin and of water-extracted muscle. Another view, however, is taken by Kaye [1924] in a more detailed examination of the nitroprusside reaction given by skin. Kaye definitely attributes the reaction to the presence of glutathione, basing this conclusion on the statement that the substance responsible for the reaction can be washed out by water. This is a result which has never been obtained by me and, in fact, one of the main reasons for associating this skin constituent with the thermostable sulphydryl constituent of muscle was its resistance to extraction by water. It is the purpose of this paper to record the observations which have been made in the course of examining the sulphydryl reaction of skin.

General Observations.

The technique for examining skin for the sulphydryl reaction has already been described [Walker, 1924]. It is most conveniently demonstrated when the skin is in section, but a rough qualitative test may be carried out upon any piece of excised skin, the reaction showing along the cut edge. Many different types of skin have been examined; viz. mouse, rabbit, cat, sheep, pig, calf and human. The reaction is essentially the same in all cases, varying in degree somewhat with variation in the skin structure. In general, skins rich in hair give intense nitroprusside reactions. In human skin the sulphydryl layer is relatively thin; the only other skin examined which is comparable to human skin in this respect is that of the pig. Nevertheless the sulphydryl layer of skin is to be found independently of the presence of hair, for it can be demonstrated in such hairless structures as the skin from the finger-tip, the female breast, and the eyelid. It is highly probable that a similar layer exists in all epithelial tissue and it is noteworthy that such a layer is readily demonstrable in the trachea (cat, rabbit), just as definitely located as in skin.

Stability.

Of all the skins examined calf-skin has proved the best for most experimental purposes and has been used throughout this investigation. The skin was clipped as free as possible of hair, and any subcutaneous tissue present was removed. The skin was used either fresh or after storage in alcohol. The skin-sections used varied in thickness between 0·25–0·5 mm. Thinner sections (20μ) had been tried but the nitroprusside reaction observed in them was only faint and such sections are especially susceptible to oxidation.

One of the earliest facts to become apparent was the general stability of the sulphydryl constituent of skin. In the first place, its post-mortem survival is very marked; skin examined three days after death gives a reaction not appreciably less intense than that of skin removed a few minutes after death. The skin may be stored in alcohol for several weeks without losing its sulphydryl reaction. It may be thoroughly dehydrated with alcohol and embedded in paraffin without its reaction being impaired. After this latter treatment it is, of course, necessary to dissolve away the wax and to steep the section in water before carrying out the sulphydryl test.

Resistance to extraction.

The sulphydryl constituent proved to be resistant to extraction with alcohol or ether. In my hands it has also proved equally resistant to extraction with water. Upon this latter point hinges the decision as to whether the sulphydryl reaction obtained is due to glutathione or not. It is certainly true that skin loses its sulphydryl reaction on prolonged immersion in water, but this is attributed to oxidation. It may be thought that the simplest way of testing for glutathione would be to extract a quantity of skin with water and to test the solution obtained for the nitroprusside reaction. This has been done several times without obtaining the slightest positive reaction in the fluid. The same negative result was obtained on extracting chopped skin with dilute sulphuric acid and with acidified saturated ammonium sulphate. It is significant that Kaye [1924] makes no mention of the extractant giving a nitroprusside reaction. In an endeavour to settle the point a number of sections (0·5 mm. thick) of fresh calf-skin were extracted six times successively with boiling water. The only effect upon the sulphydryl layer was to intensify slightly the nitroprusside reaction. Again, a number of similar sections were thrown into distilled water,,previously boiled and cooled, covered with a layer of toluene and allowed to stand. Sections were removed and tested each day. The sulphydryl reaction was present until the fourth day. Thereafter it could be shown that the oxidised compound (the —S—S—form) was still present in the skin at the end of 14 days. The test for the disulphide form is a modification of the ordinary nitroprusside reaction and is described in the preceding paper.

Oxidation.

The general stability of the sulphydryl layer has already been discussed. In particular, it is noteworthy that the layer shows considerable resistance to atmospheric oxidation. For example, a number of moist skin sections, 0·3 mm. thick, were spread upon a strip of suspended muslin and were thus freely exposed to the air. Although the sections had shrivelled up within a few hours the sulphydryl reaction could still be demonstrated in them (after soaking them in $M/120$ acetic acid to restore their shape) after 72 hours. In the thinner (20–30μ) sections used by Kaye it is natural that oxidation should be more rapid. Even the thermostable sulphydryl constituent of muscle succumbs to oxidation when the muscle powder is exposed to the air in fine layers [Dixon and Hopkins, 1922]. Further, according to Kaye [1924], the sulphydryl layer of skin is "...stable to some degree against alkalies (lime-water and ammonia)...." This does not suggest glutathione.

One further point of interest arose in examining the effect of allyl isothiocyanate on the sulphydryl layer of skin. Treatment of skin *in vitro* with allyl isothiocyanate, either by painting it on the surface of the skin or by shaking up sections with an aqueous solution of the oil, causes complete disappearance of the sulphydryl reaction very rapidly. The skin so treated no longer gives a disulphide reaction, so that we are not dealing with a case of oxidation but probably of combination. Muscle thoroughly extracted with water is similarly affected by allyl isothiocyanate.

Altogether it appears more reasonable to associate the sulphydryl layer of skin with the thermostable sulphydryl constituent of muscle rather than with glutathione. It is not unlikely that small amounts of glutathione are present normally in the sulphydryl layer of skin, analogous to the presence of glutathione in muscle.

SUMMARY.

No evidence has been obtained to show that the nitroprusside reaction of skin is due to the presence of glutathione. It is suggested that the reaction is due to the presence of a substance similar to (or identical with) the thermostable sulphydryl constituent of muscle.

It has been found that skin rapidly loses its sulphydryl reaction under the influence of allyl isothiocyanate.

I am grateful to Professor Peters for his interest and helpful criticism.

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CLVI. STUDIES OF THE BACTERIAL DECAY OF TEXTILE FIBRES.

II. A PRELIMINARY STUDY OF THE DETERIORATION OF SAMPLES OF ARTIFICIAL SILK THROUGH THE ACTION OF MICRO-ORGANISMS.

By AAGE CHRISTIAN THAYSEN and HENRY JAMES BUNKER.

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(*Received October 27th, 1925.*)

The investigations described in this report comprise a number of preliminary exposure tests of four different types of artificial silk to the action of micro-organisms, made with a view to determining their rate of decay. The existing literature contains only one reference to the behaviour of artificial silk when exposed to the action of micro-organisms. In this reference Dorée [1920] reports that samples of cellulose acetate silk were exceptionally resistant to the destructive action of the cellulose-decomposing micro-organisms of sea water. In view of the growing importance of the artificial silk industry, the extension of these observations to other types of artificial silk, as well as to other forms of cellulose-decomposing micro-organisms, particularly those of the soil, was thought sufficiently important to justify a renewed enquiry.

The experiments reported in the following pages should not be regarded as an exhaustive answer to this enquiry but rather as of a preliminary nature.

The available material was of a very heterogeneous nature and consisted partly of skeins of artificial silks prepared by the viscose process, the Chardonnet process and the cuprammonium process, and partly of two types of fabric, a lighter and a heavier, woven from cellulose acetate silk, which is known commercially as "Celanese." Since the number of filaments in the threads of the samples varied considerably and the thickness of the various filaments showed considerable divergencies[1] and since, also, some of the samples were

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[1] The number of filaments in the threads were:

Viscose	21
Nitro	19
Cuprammonium; more than ...	40
Celanese (lighter) (warp and weft)	17
,, (heavier) ,,	39

The *relative* diameters of the filaments were:

Viscose	0·9
Nitro	1·0
Cuprammonium	0·4
Celanese	0·6

in the form of skeins, and others made up into fabrics, it will be realised that great care had to be shown in the interpretation of changes observed in the tensile strength of the samples during exposure. Furthermore, it had to be borne in mind that some of the silks might lose more of their tensile strength than others on prolonged contact with moisture, quite irrespective of the changes caused by micro-organisms.

For the above reasons it was thought preferable when measuring the resistance of the various samples to rely on changes in the macroscopic and microscopic appearance of the filaments and on the number of micro-organisms present on them during and after exposure, rather than on changes in their tensile strength.

It is realised that these criteria are not entirely satisfactory, since they indicate the more pronounced changes only. They are, however, on that account safer to adopt for such heterogeneous material as that used than the determination of changes in the tensile strength of the filaments.

The exposure tests carried out may be divided into three groups: *A*, tests in which the samples were placed anaerobically in a food solution favouring the development of cellulose-decomposing bacteria; *B*, tests in which the samples were fixed in square frames buried vertically in a light garden soil and reaching to a depth of 30 cm., thus ensuring a thorough exposure to the action of aerobic and anaerobic cellulose-decomposing micro-organisms of the soil; *C*, tests in which the samples were fixed in square frames and submerged in sea water, conditions which in previous experiments had been found to ensure exposure to the action of the cellulose-destroying micro-organisms of the sea.

The observations made in these various tests are recorded below in tabular form.

Series A. Rate of destruction of artificial silks exposed to the action of a culture of cellulose-decomposing micro-organisms.

In this series the various samples were placed in 500 cc. flasks filled with a medium consisting of peptone 0·1 %, di-potassium hydrogen phosphate 0·1 %, magnesium sulphate 0·1 %, sodium chloride 0·1 % and ammonium sulphate 0·2 %. Each flask was inoculated with 2 cc. of a culture containing anaerobic cellulose-decomposing bacteria and incubated at 37°.

Under the conditions of this series of experiments the cellulose acetate silk and the nitro silk were probably not attacked by the anaerobic cellulose-decomposing bacteria used. The sample of viscose silk showed less resistance to these forms than the first two mentioned but was still considerably more stable than the cuprammonium sample, which deteriorated completely within a fortnight, an even faster rate than that shown by a sample of raw cotton, of Indo-American origin, which, during that time, had been damaged to 57 %, estimated by the swelling test [Fleming and Thaysen, 1921].

Table I.

Days' exposure at 37°	Cuprammonium silk	Viscose silk	Chardonnet silk (nitro silk)	Celanese silk (cellulose acetate silk)
5	Sample is reduced to a pulp. The filaments show bad fraying. Sulphuretted hydrogen is evolved	Sample is yellowish, discoloured. The filaments show some fraying. Sulphuretted hydrogen is evolved in small quantities	Sample is not discoloured; there is no evidence of damage to the filaments. Sulphuretted hydrogen is evolved	No change
13	Only few filaments remain. Sample discarded	Sample is discoloured and slimy. The edges of the filaments are more jagged than before	Sample still undamaged	No change
19	—	Little change	Sample still undamaged	No change
26	—	The filaments are getting appreciably weaker and their edges more jagged	No change; re-inoculated with a fresh culture of cellulose-destroying micro-organisms	No change; re-inoculated with a fresh culture of cellulose - destroying micro-organisms
33	—	No further change; re-inoculated	No change; re-inoculated	No change; re-inoculated
40	—	The filaments now form a slimy mass	No change; re-inoculated	No change; re-inoculated
47	—	Little remains of the filaments. Sample discarded	No change; re-inoculated	No change; re-inoculated
103	—	—	Some of the filaments feel a little weaker and are discoloured slightly yellow; re-inoculated	No change; re-inoculated
138	—	—	Though weaker, the filaments show no microscopic signs of decay; re-inoculated	No change; re-inoculated
200	—	—	No further change	No change

Series B. Rate of destruction of samples of artificial silk buried in a light garden soil.

The rate of decay of the samples exposed in the soil was of the same order as that observed in the laboratory anaerobic experiments, the cuprammonium sample going first, the viscose sample next. In the soil, however, the nitro sample was also affected and its resistance was only a little greater than that of the viscose silk. The cellulose acetate silk remained unaffected even after 30 weeks' exposure. When examined after 27 weeks, the heavier cellulose acetate fabric showed a greenish patch about 5 cm. from the top. On microscopic examination this was found to be due to a green coenocytic filamentous alga. The fabric was not damaged, however, and remained normal throughout the test. That this resistance was not due to the absence of cellulose-decomposing micro-organisms was shown experimentally by the inoculation of flasks, such as those already described, containing filter paper and food solution with pieces of the exposed fabrics added. The filter paper of these flasks was invariably found to be decomposed after 8–14 days' incubation.

Table II.

Weeks' exposure in the soil	Cuprammonium silk	Viscose silk	Nitro silk	Celanese silk Light and heavy fabric
2	No change	No change	No change	No change
3	Some of the filaments show frayed edges and on gentle pulling the threads break at a spot about 15 cm. from the surface of the soil	Many of the filaments beginning to fray at the edges. The threads are discoloured pink	Some of the filaments beginning to fray at the edges	No change
4	The filaments show much fraying. The threads break fairly easily on gentle pulling	The fraying has not advanced very much. The threads are nevertheless very easily broken. The pink discoloration is intensified	Many of the filaments now show fraying at the edges. The sample is nevertheless still strong and does not break easily	No change
5	The fraying at the edges of the filaments has increased. The threads break at the slightest touch	No appreciable change	No appreciable change	No change
6	In a very poor condition; the bulk of the sample is a slimy mass. Sample discarded	Slightly more pronounced fraying at the edge of the filaments	Perhaps slightly more fraying at the edges of the filaments	No change
7	—	The filaments are more markedly frayed and the threads break easily on gentle pulling. The threads feel slimy	Stronger than the viscose sample; the fraying of the edges of the filaments is more marked than last week	No change
8	—	Markedly weaker. The filaments are now a slimy mass. Sample discarded	The threads are fairly resistant though the fraying of the edges of the filaments has increased	No change

The various samples exposed in the soil were subjected to a bacteriological analysis on each examination in order to determine whether an increase in the secondary micro-flora of these samples occurred during exposure. As is known, it is not possible, or at least is very difficult, to isolate cellulose-decomposing bacteria in pure culture; the enumeration of these organisms on a given sample of material is therefore not feasible. But an increase in the readily cultivable secondary micro-flora, which under natural conditions is associated with the cellulose-decomposing bacteria and lives on their decomposition products, cellobiose and glucose, indicates an activity of the latter. A sudden rise therefore in the numbers of this secondary flora can generally be regarded as a sign of an increased activity of the cellulose-decomposing micro-organisms, particularly when no other cause for an increase is apparent, such as the addition of food materials or seasonal changes in the soil flora.

For the bacteriological analyses 1 g. of the material to be examined was shaken vigorously for 2 hours with 9 cc. of physiological saline solution and this standard suspension plated out on the following media: ordinary nutrient agar, wort agar, cellulose agar prepared according to the formula of McBeth and Scales [1913], nutrient gelatin, and glucose agar under anaerobic conditions (Burri tubes).

The result of these analyses showed that there was a marked rise in the numbers of the secondary micro-flora in each case, the rise being noticeable first in the cuprammonium silk, a week later in the viscose silk, and three weeks later in the nitro silk. A slight increase in the secondary micro-flora of the cellulose acetate silk took place nine weeks after the increase had occurred in the cuprammonium silk.

The presence of both aerobic and anaerobic cellulose-decomposing bacteria on the various samples of exposed artificial silks was confirmed experimentally by inoculating samples of the exposed silks into a medium containing filter paper and suitable for the growth of cellulose-fermenting bacteria. In every case, even where exposed Celanese silk was used, the filter paper became decomposed.

Series C. Rate of destruction of samples of artificial silk submerged in sea water.

The samples of series *C* were fixed in square aluminium frames and submerged in the sea at the jetty of the Royal Naval Cordite Factory, at a place where previous investigations had shown cellulose to become readily destroyed.

In Table III (p. 1093) the observations made in this series of exposure tests are recorded.

The rate of decay of all samples was markedly faster in the sea than in the soil. This on the whole confirms previous observations made with other types of textiles. More noteworthy is the fact that the difference in the rate of decay between the first three artificial silks named has been almost completely obliterated in the sea exposure tests. The only marked resistance to decay is that observed in the cellulose acetate samples. The enumeration of the secondary micro-flora present on the decaying samples of this series offered little information of interest, and did not show the increase observed in the case of the samples buried in the soil. It was established that both aerobic and anaerobic cellulose-decomposing micro-organisms were present on all the submerged samples which showed definite signs of decay. Before such decay had commenced, cellulose-decomposers were absent from the textiles.

CONCLUSIONS.

From the experimental data collected in the three series of exposure tests it may fairly safely be concluded that the samples of cellulose acetate silk tested showed the greatest resistance to micro-biological destruction. Dorée's observations have thus been confirmed. Evidence has also been obtained of a varying degree of resistance among the other types of artificial silk examined, the order of merit of the samples tested being nitro silk, viscose silk and cuprammonium silk.

Table III.

The temperature of the water varied between 11·5°–14·0° *during the exposure trials.*

Weeks' exposure of samples in the sea	Cuprammonium silk	Viscose silk	Nitro silk	Cellulose acetate silk	
				Light fabric	Heavy fabric
1	Most of the filaments show frayed surface	Same as cuprammonium sample	Same as cuprammonium sample	No change	No change
2	The fraying of the surface of the filaments is much more marked. The threads break fairly easily	The fraying of the surface of the filaments has increased but the sample does not break as readily as the cuprammonium sample	Same as viscose sample	No change	No change
3	Sample almost completely destroyed	The destruction of the filaments has progressed; the sample is however distinctly superior to the cuprammonium sample	Same as the viscose sample	No change	No change
4	Sample reduced to a slimy mass	Sample almost completely decomposed	Same as the viscose sample	No change	No change
9	—	—	—	No change	A few of the filaments show frayed surface
10	—	—	—	Though the fabric tears fairly readily there is no sign of damage to the filaments	The number of filaments showing frayed surface has increased
11	—		—	No further change	Considerably worse. The damage to the filaments is more pronounced and the fabric tears readily
12	—	—	—	Both samples are now badly decayed. The thinner fabric is still superior to the heavier and shows a considerable number of normal filaments	

In endeavouring to account for this difference, it strikes the observer that the most resistant form of artificial silk is that in which the cellulose is present as a fully saturated ester. The sample next to this, the nitro silk, is one in which the cellulose had been at one time a fully saturated ester. In the viscose silk the cellulose may have been esterified but certainly not completely. In the least resistant sample, the cuprammonium silk, the cellulose was present in a hydrated form which had not in any way been chemically combined with acids. It would appear desirable to determine how far a preliminary esterification of cellulose offers a means of protecting it against micro-biological deterioration.

Another point which has undoubtedly to be considered in endeavouring to elucidate the difference in resistance shown by the various artificial silk samples, is their crystalline nature. Of the four types included in the experiments the cellulose acetate silk has been definitely shown to be of an amorphous nature [Herzog and Jancke, 1920], whilst cellulose precipitated from cuprammonium solution and viscose silk are both crystalline, like untreated cellulose. The structure of nitro silk is, it is believed, as yet undetermined.

In this connection it is interesting to recall that a difference in resistance to enzymic decomposition was observed by Pringsheim and Aronowsky [1922] between ordinary inulin and inulin which had been obtained from inulin acetate by hydrolysis. Of these, the former was crystalline and the latter amorphous.

It is intended to continue the study of the resistance of artificial silks from the two above-mentioned points of view.

SUMMARY.

The rates of destruction of four different types of artificial silks on exposure to the action of micro-organisms have been studied. It has been established that the rate varies considerably, being slowest in the case of cellulose acetate silk and quickest in the case of cuprammonium silk.

This difference may possibly be due either to physical or to chemical differences in the various types of artificial silk.

The above experiments were carried out on behalf of the Fabrics Co-ordinating Research Committee of the Department of Scientific and Industrial Research at the Bacteriological Laboratory of the Royal Naval Cordite Factory, Holton Heath. The authors desire to express their thanks to the Department of Scientific and Industrial Research and to the Admiralty for permission to publish the results obtained.

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CLVII. THE FATE OF THE BLOOD SUGAR AFTER INSULIN INJECTION IN NORMAL ANIMALS.

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Introduction.

What becomes of the blood-sugar which disappears in such large amounts after the injection of insulin still remains an unsolved problem [Macleod, 1925].

Though it is recognised that the red blood corpuscles play an important part in many physiological functions, no one has expressed the view that they might occupy an equally important rôle in the mechanism of the action of insulin. That this might be so, however, was suggested by the results of certain investigations, to be communicated later, which have been in progress in this laboratory for some time. Briefly stated, our view was that insulin in the presence of the erythrocytes of living blood caused glucose to combine with certain of the other blood constituents, thereby forming a non-reducing complex probably containing both nitrogen and phosphorus, which thereafter found its way to all the tissues of the body, more especially to the liver and muscles, to be utilised or stored as required. This would naturally be a normal physiological process; but on the injection of insulin in a normal healthy animal the reaction would be so accentuated that though the reducing property of the whole blood or of the blood-plasma would be greatly diminished, the blood itself would still contain the greater part of the sugar, which, being now present in a combined state, would only be detected on hydrolysis.

To test the particular point in question a series of experiments was planned, and while these were in progress Scott and Best [1924] published a communication, in which they suggested "that the mechanism for the rapid disappearance of sugar from the blood is to be found in the blood itself." They report, contrary to the observations of McCormick, Macleod, Noble and O'Brien [1923], that while the reducing power of the blood of normal animals is usually only very slightly increased by hydrolysis, that of animals rendered hypoglycaemic by insulin, when similarly hydrolysed, shows a pronounced increase in reducing power, being equal to 70 % of that of the original normal blood. As no details of experimental results or methods have been published by these workers, it is difficult to say whether or not our findings are in agreement.

EXPERIMENTAL.

The majority of the experiments were carried out on rabbits. The animals used were deprived of solid food for 16–20 hours, and were all of approximately the same weight, about 2·9 kg. The blood was removed from the ear vein by means of a 20 cc. Record syringe and transferred immediately to two centrifuge tubes of approximately 10 cc. capacity containing a trace of finely powdered potassium oxalate to prevent coagulation.

A number of capillary tubes, usually four, were then charged with blood for the determination of the relative volume of corpuscles and plasma by the method described by Harvey [1920].

After the normal blood samples were drawn, the animals were injected subcutaneously with comparatively large doses of insulin (15–20 units). Two hours later, or on the appearance of convulsions, samples of blood were again removed from the ear vein as described above.

Determination of sugar content of plasma and corpuscles.

With the minimum of delay the tubes containing the blood samples were centrifuged for 10 minutes at approximately 4000 revolutions per minute. It was found that under this treatment the corpuscles and plasma are satisfactorily separated, as further centrifuging did not alter the relative volumes. By means of a capillary pipette the plasma was then removed carefully and as completely as possible without disturbing the corpuscular layer, following the method of Wu [1922], and the sugar content of the plasma and of the corpuscles determined by MacLean's method [1919].

In connection with the determination of the sugar content of the corpuscles it was found in the preliminary experiments that complete laking of the corpuscles by water previous to the addition of the sodium sulphate solution did not materially affect the results. Consequently in the later experiments the corpuscles were treated exactly as whole blood.

The reducing power of protein-free filtrates after hydrolysis.

At the same time, protein-free filtrates obtained by MacLean's method from the plasma and also from the corpuscles were hydrolysed by heating with sulphuric acid for half an hour under a reflux condenser, 1 cc. of 25 % sulphuric acid being employed for every 20 cc. of filtrate. After cooling, the sulphuric acid was exactly neutralised by the addition of the necessary volume of a concentrated solution of sodium carbonate, and the sugar content then determined by means of MacLean's copper reagent.

It may be well to add that in the first experiments on the determination of the sugar content of protein-free filtrates after hydrolysis, the results obtained were very high. This was traced to the use of an excess of sodium carbonate, so that, although MacLean's process furnishes us with an excellent method for the determination of sugar in whole blood, care must be taken in applying this and similar methods to other work where the conditions may be somewhat altered. The recent paper by Quick [1925] on the effect of sodium carbonate concentration in the Benedict sugar method is pertinent in this connection.

The reducing power of plasma and corpuscles after hydrolysis.

Samples of plasma and of corpuscles were also hydrolysed directly as follows: 1 cc. plasma (or corpuscles) was added to 99·5 cc. sodium sulphate solution, 5 cc. 25 % sulphuric acid added, and the mixture refluxed for 1 hour. It was then cooled, exactly neutralised with 14·5 cc. sodium carbonate solution, and, after the effervescence had ceased, treated with 5 cc. of "dialysed iron." After thorough shaking, the mixture was filtered through a Whatman No. 40 paper of 9 cm. diameter, and 20 cc. samples of the filtrate used for the sugar estimation.

No difficulty was experienced in obtaining water-clear filtrates from either plasma or corpuscles, but while no irregularity resulted with filtrates from plasma, those from the corpuscles invariably gave slight biuret reactions and consequently contained small amounts of protein material. As this could not be removed by modifying the conditions of the precipitation, it was evidently of the nature of a proteose or peptone—probably derived by hydrolysis of the histone, globin. The determination of the reducing power of the corpuscles after direct hydrolysis is thus rendered unreliable, and it is difficult to estimate how far the presence of, say, peptone, interferes with the process. Tests with Witte's peptone proved that high values would result in determining the reducing power of a solution either by MacLean's or Hagedorn and Jensen's methods [1923], if peptone were present to the extent of 0·03–0·05 %. Consequently this source of error should be guarded against in work of this type.

In order to make clear the discussion which follows, the results of some typical experiments are given in Table I. As already indicated, the majority of the experiments were conducted on rabbits, but similar results were also obtained with rats.

Table I. *Normal rabbit before insulin.*

	A		B		C		C − A	
Animal no.	Plasma %	Corps. %	Plasma %	Corps. %	Plasma %	Corps. %	Plasma %	Corps. %
2	0·136	0·050	—	—	0·136	0·0634	0·0	0·013
3	0·166	0·040	0·170	0·076	0·166	0·096	0·0	0·056
Normal rabbit after insulin.								
2	0·044	0·030	—	—	0·045	0·079	0·001	0·049
3	0·036	0·038	0·060	—	0·039	0·125	0·003	0·087
4	0·053	0·039	0·063	0·067	0·058	0·106	0·005	0·066

(A = before hydrolysis. B = after hydrolysis of "protein-free" filtrate. C = after direct hydrolysis.)

Further, as the relative volumes of plasma and corpuscles had been determined in most cases, it was possible to calculate the values for the whole blood. These are given in Table II.

Table II. *Sugar content of whole blood before and after hydrolysis.*

Animal	Before hydrolysis %	After hydrolysis %	Increase %
1. Normal rabbit	0·136	0·155	0·019
2. ,, ,,	0·107	0·112	0·005
3. ,, ,,	0·122	0·141	0·019
4. ,, ,,	0·128	—	—
,, rat	0·093	0·110	0·017

Average increase = 0·015 %.

Animal	Before hydrolysis %	After hydrolysis %	Increase %
2. Insulin rabbit	0·039	0·056	0·017
3. ,, ,,	0·037	0·068	0·031
4. ,, ,,	0·049	0·071	0·022
,, rat	0·057	0·113	0·056
,, ,,	0·025	0·055	0·030

Average increase = 0·031 %.

DISCUSSION OF RESULTS.

Changes in the whole blood. The results indicate that the sugar content of the blood in the case of the normal rabbit or rat is only slightly increased on hydrolysis. In animals rendered hypoglycaemic by insulin the actual increase in reducing power brought about on hydrolysis is, on the average, about twice as great as that obtained with normal animals. When due account is taken of the usual increased reduction after hydrolysis, apparently 14–20 % of the sugar disappearing from the blood would be accounted for.

Influence of insulin on corpuscle sugar and plasma sugar. Inspection of the results in Table I show that the percentage of free reducing material in the corpuscles is very small and remains practically unchanged before and after the injection of insulin. In fact, this material would seem to correspond very closely to the non-glycolysable and non-fermentable reducing substances recently proved to exist in blood by Hiller, Linder and Van Slyke [1925]. The plasma sugar, on the other hand, has a comparatively high initial value, and on the administration of insulin, falls very nearly to the value of that present in the corpuscles.

Changes on hydrolysis. On hydrolysis the plasma sugar remains practically unaltered in both the normal and insulin-treated animal, whereas the corpuscle sugar increases in both cases. This behaviour is contrary to expectation and points to the fact that if a complex non-reducing compound be formed as the result of the injection of insulin, the compound does not remain in the plasma, but may, to a certain extent, be associated in some manner with the corpuscles.

As only about one-sixth of the vanished sugar can thus be accounted for, our original hypothesis cannot be maintained, and it seems more likely that a non-reducing sugar complex is formed in the blood and immediately distributed among all the tissues of the body. It is also probable that the different kinds of tissue may be able to hold varying amounts of the complex, so that liver and muscle tissues may take up a greater proportion than other tissues. The red blood corpuscles are thus regarded as a type of tissue, and consequently they would get their share of the glucose which disappears from the blood-plasma.

Whether the results of our experiments support this view is unfortunately rendered doubtful, for at this stage the analyses become unreliable because of the simultaneous production and interfering action of hydrolysed proteins. In the experiments under consideration, however, the error introduced should be of the same order in the case of blood taken in the normal and in the hypoglycaemic condition, and as, so far, greater values have always been obtained in the latter, it seems possible that the difference may be due to the action of insulin. The amount of this difference is not large, and though well beyond the usual experimental error, may still be accounted for in this way; but it must be remembered that if the view indicated above be correct, only a small difference could possibly result. One hesitates, however, to draw the definite conclusion that insulin brings about the conversion of the glucose present in the plasma into a non-reducing complex, which then becomes combined in some manner with the tissues, amongst which are included the red blood corpuscles. The results communicated in this paper, if not conclusive, are, at least suggestive, and the work is being continued in the hope that it may help to solve the mystery of what is the most interesting and fundamental problem in connection with the action of insulin.

SUMMARY.

1. The reducing power of the corpuscles of the blood of the normal rabbit is comparatively low, and is not influenced to any extent by the injection of insulin.

2. The reducing power of the plasma of the blood is comparatively high, and under the action of insulin falls to the level of that possessed by the corpuscles.

3. On hydrolysis, the plasma sugar is little affected either before or after insulin, whereas the reducing power of the corpuscles increases in both conditions.

4. Part of the increase in reducing power of the corpuscles after hydrolysis is due to the interfering action of peptone produced by the hydrolysis; but, as the increase is greater after insulin than in the normal condition, it suggests that a non-reducing glucose complex may be formed which attaches itself in some way to the tissues, including the red blood corpuscles.

5. About a sixth part of the sugar disappearing from the plasma after insulin may be accounted for by the erythrocytes acting in this manner, but no definite conclusion can be drawn.

6. It is pointed out that certain experimental difficulties may arise in applying the usual methods of estimating sugars to "hydrolysed" solutions.

All the animal experiments necessary for this work were carried out by Professor Herring, to whom I am greatly indebted not only for this service but also for much valuable advice throughout the research.

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CLVIII. STUDIES ON THE METABOLISM OF ARGININE AND HISTIDINE.

PART II. ARGININE AND HISTIDINE AS PRECURSORS OF PURINES.

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(*Received November 2nd, 1925.*)

In the first paper of this series [Stewart, 1925] it was reported that liver perfusion experiments had failed to afford any evidence in support of the suggestion, advanced by Ackroyd and Hopkins [1916], that arginine and histidine are utilised in the animal body as precursors of purines. As was there pointed out, however, the evidence derived from the perfusions, while unable to confirm the hypothesis, was equally unable to invalidate it, and so did nothing to remove the uncertainty as to the function of arginine and histidine which has been produced by the contradictory results of subsequent workers.

It appeared, therefore, that a repetition of the work of Ackroyd and Hopkins might be useful to confirm the experimental basis on which their conclusions were founded. Further, such a series of experiments might throw some light on the cause of the contradictory results which have been published. For it is noticeable that, whereas Ackroyd and Hopkins and also Harding and Young [1919], who obtained similar results, employed young growing animals in their experiments, others, like Lewis and Doisy [1918], who failed to find any connection between purine excretion and histidine and arginine intake, employed adult animals. Indeed, Harding and Young remark, as a possible explanation of the discrepancies, that in the adult animal there may be a cessation or serious impairment of many synthetic processes which are active in the young. There already exists, of course, some evidence for such a state of affairs in the case of tryptophan. Although, as has been abundantly proved by the work of Hopkins and Willcock [1907] and others, young animals are unable to maintain growth on a tryptophan-free diet, Osborne, Mendel and Ferry [1912] have succeeded in keeping adult animals for 178 days on a diet containing no tryptophan without appreciable loss of weight.

The feeding experiments reported in this paper were thus undertaken for a twofold purpose: to confirm the experimental foundation of the suggestion that histidine and arginine are used as purine precursors and are interchangeable in metabolism, and, by comparing the behaviour of adult and growing animals, to attempt an explanation of the divergent results which have been obtained hitherto.

EXPERIMENTAL.

Four experiments, in each of which two rats were used, are described. The animals were all bred from Wistar Institute stock, and were all females. In Exps. 1 and 2, which were designed to test the effect of arginine and histidine deficiency on adult animals, the four rats were from the same litter, and had passed the menopause. In Exps. 3 and 4, again, all four rats were from the same litter, but were started on the experimental diet when their weight was nearly 50 g. The pairs of rats were kept in cages of the type described by Ackroyd and Hopkins [1916], and the temperature was kept as constant as possible. For the greater part of the time it remained at about 65° F., but occasionally, during the hot weather, it rose to 70° F. The animals, however, did not appear to suffer and continued to eat well.

For the collection of the urine and removal of contaminating faeces and food particles, the apparatus described by Ackroyd and Hopkins was employed. The funnel in which the cage stood was washed down with warm water several times each day, the urine and washings being at once transferred to a stoppered flask containing a little chloroform and sufficient acetic acid to keep the mixture definitely acid. The flask was kept in an ice-chest and the week's collection made up to a convenient volume—usually 500 cc.—for analysis.

The total nitrogen in the urine was determined by a micro-Kjeldahl method. After removal of ammonia, Wiechowski's method [1913] was employed for the determination of allantoin. In the final stage, however, after precipitation of the allantoin by the mercuric acetate reagent, the nitrogen content of the precipitate was determined by the Kjeldahl method, a procedure which preliminary experiments had shown to be reliable.

The diets used were prepared in the manner described by Ackroyd and Hopkins. In the control diet the percentage of nitrogen (2·1) was rather lower than in the particular example given by these authors (3·12), although they state that they used diets containing as little as 2·0 % of nitrogen. Tryptophan was added to the extent of 1·5 %, and cystine to 0·5 % of the weight of the caseinogen originally taken for hydrolysis.

The vitamin supply was drawn partly from the fresh butter used to provide the fat of the diet, and partly from marmite, a commercial yeast extract. The marmite was ground with plaster of Paris and extracted with absolute alcohol. Since the extract contained purines and amino acids, it was evaporated to dryness and the residue dissolved in a little water. Silver sulphate was added in quantity for complete precipitation first in acid, and second in alkaline solution (baryta was used in making the mixture alkaline).

After complete removal of silver, barium, and sulphate ions, the solution, now free from purines, and giving no more than a trace of colour with diazobenzenesulphonate, was evaporated to a syrup, which was mixed with starch and, after drying in a vacuum desiccator, incorporated into the diet in the proportion of 5 g. of original marmite to 100 g. of original caseinogen. No doubt, in the silver precipitations a certain amount of vitamin was carried down, but, as control experiments showed, the supply was adequate for satisfactory growth.

The removal of histidine and arginine from the hydrolysed caseinogen was accomplished by the silver precipitation method of Kossel and Kutscher, using silver sulphate and making alkaline with baryta. After quantitative removal of silver, barium, and sulphate ions the solution was evaporated *in vacuo*. When care was taken to add a liberal excess of silver, the resulting syrup was free from all but the merest traces of arginine and histidine. Samples of the syrup corresponding to 1 g. caseinogen, dissolved in 5 cc. water, gave neither the diacetyl test for arginine nor the diazobenzenesulphonate test for histidine.

In those diets from which only one of the diamino acids was to be absent, the other was restored to the silver-precipitated mixture. For every 100 g. original caseinogen, 2 g. amino acid were added, *i.e.* approximately the amount present in the protein. No attempt was made to discover the minimum amount of either histidine or arginine requisite for maintenance or growth, and the amount actually added supplied the animals with a very large excess over the quantity which would be needed to produce the allantoin excreted.

Throughout the experiment the animals received a diet containing the same proportion of fat, carbohydrate and nitrogen. The diets at the different stages of the experiment differed only in the presence or absence of arginine and histidine. The consumption of food was good throughout, and though the daily consumption per pair of rats varied by a gram or two, the weekly averages were fairly uniform and showed no tendency either to fall or rise during the critical periods of the experiments. Thus in Exp. 4 (rats VII and VIII) the daily food consumption per rat varied only from 5·8 to 6·7 g.

RESULTS AND DISCUSSION.

1. *The effect of histidine and arginine deficiency on young rats.*

Fig. 1 shows the rate of growth of the young animals during the experimental period. At first, while the control diet containing a complete supply of amino acids was being given, growth proceeded at a rate which, though rather less than normal, may be regarded as satisfactory. Since each curve gives the combined weight of two rats, the average daily increase per rat was about 0·75 g. On a diet of bread and milk, similar rats were growing at the rate of about 1 g. per day. The withdrawal of histidine and arginine brought about a complete change. At once the animals ceased to grow in spite of an undiminished food consumption, and during the period in which this deficient diet was being given, they lost weight at an average rate of 0·4 g. per day. When histidine was restored to the diet of rats VII and VIII, growth was resumed and proceeded at a rate which was unaltered when arginine was restored as well, so that the animals were once more receiving the complete amino acid mixture.

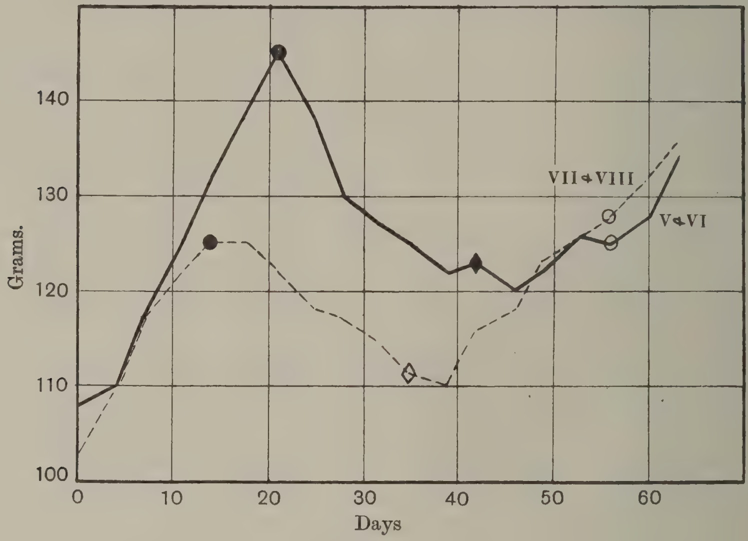

Fig. 1.

● Arginine and histidine removed from diet.
○ Arginine and histidine restored to diet.
◆ Arginine alone restored to diet.
◇ Histidine alone restored to diet.

The case of arginine is not so clear. During the period when this amino acid was present in the diet while histidine was absent (rats V and VI), there was a preliminary fall in weight—or rather continuation of the fall—followed by a definite rise over a week and a second slight fall. The net result of a fortnight's feeding on this diet was a very slight increase in weight, very much less than the increase during the similar period in the case of rats VII and VIII. The restoration of the complete amino acid mixture was again accompanied by satisfactory growth. This result does not support that of Ackroyd and Hopkins who suggested that arginine and histidine are essentially equivalent—*i.e.* interchangeable—in metabolism. Certainly the method of carrying out the experiments differs in one respect from that employed by Ackroyd and Hopkins, although the difference does not appear to be significant. Their animals, after a period on the control diet, received a diet which contained arginine but not histidine (or *vice versa*); they never, as in the experiments reported in this paper, had one or other of the amino acids restored to a diet deficient in both.

The fact that on the restoration of arginine to the diet of rats V and VI the fall in weight ceased and the animals succeeded in maintaining themselves at the lower level (if not in growing, for, if one neglects the fall immediately after the resumption of arginine feeding—a fall which occurs also in the case of rats VII and VIII when histidine was first restored—there is a definite increase of weight) may perhaps be significant. It is not in agreement with the observation of Rose and Cox [1924] who found that arginine, as carbonate or nitrate, entirely failed to prevent the loss in weight. That statement is absolutely definite, and quite justified by their experimental observations, but so is the exactly opposite one of Ackroyd and Hopkins.

The three results are not explicable on the assumption that varying amounts of arginine had escaped precipitation in the preparation of the "deficient" diets. There is no test for arginine comparable in delicacy with the diazobenzenesulphonate test for histidine, and, as Rose and Cox point out, the silver salt of arginine is more soluble than that of histidine, so that it is probable that traces, at any rate, of arginine remained in the deficient diets Nevertheless, if lack of arginine be partly responsible for the fall in weight which does actually occur when it and histidine are withdrawn from the diet (or if it and histidine are interchangeable), it follows that the residual minute amount cannot be sufficient for the animal's needs, and that, consequently, there should be some resumption of growth when arginine is restored; in no case should the fall in weight continue. Such fall in weight can only mean that arginine is not concerned in the nutritional failure which is being manifested, in which case the completeness or otherwise of its removal is unimportant.

Contamination with histidine of the arginine used by Ackroyd and Hopkins and by the author would, of course, explain the results at once, but seems extremely unlikely. Certainly in the sample of arginine used in these experiments there was no admixture of histidine (as shown by the absence of any coloration with diazobenzenesulphonate) and Hopkins has stated that his supply was similarly pure.

For a full solution of the problem more data appear to be required. The essential question in the author's experiments is whether the administration of arginine was the factor responsible for the resumption of maintenance, or whether, in the absence of arginine, the loss of weight would still have ceased. Experiments on this point are in progress. Ackroyd and Hopkins point out a tendency, in their experiments, to the attaining of an equilibrium after a period of arginine and histidine deficiency, stating that it may possibly be due to slightly incomplete removal of these substances. Nevertheless, in all their experiments the rats continued to lose weight over a period of 4 weeks during which they received only such traces of arginine and histidine as had escaped precipitation. In the experiments of Rose and Cox the loss of weight continued, though later at a diminished rate, for much longer periods. Hence, although a definite conclusion must be withheld until the termination of the experiments now in progress, there does seem, in the growth curve of rats V and VI, to be an indication that arginine deficiency plays some part in the nutritional failure which follows the removal of it and histidine from the diet.

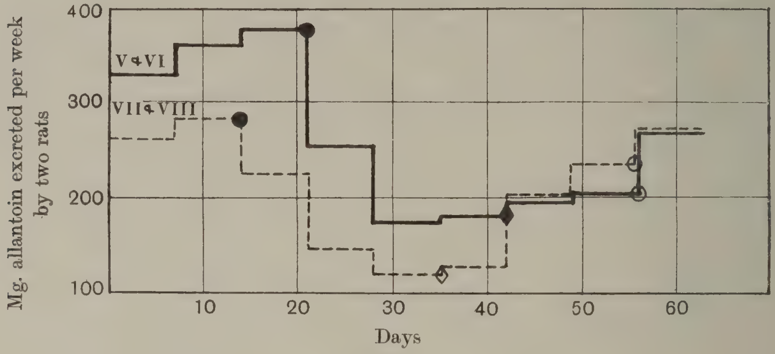

Fig. 2.

● Arginine and histidine removed from diet.
○ Arginine and histidine restored to diet.
◆ Arginine alone restored to diet.
◇ Histidine alone restored to diet.

Fig. 2 shows the weekly excretion of allantoin by each pair of rats during the experimental period. When the rats were receiving the control diet, and were growing at a satisfactory rate, the allantoin excretion increased slightly with the weight—a result in accordance with that published by Rose and Cook [1925] while this work was in progress. When arginine and histidine were withdrawn, the allantoin excretion rapidly fell, at a rate proportionately much greater than did the weight, until in the third week of the deficient diet it reached a value almost half the original. Restoration of histidine to the diet (rats VII and VIII) was followed by a rise in the allantoin excretion accompanying the resumption of growth. So far the results are in complete agreement both with those of Ackroyd and Hopkins and of Rose and Cook. The results following the restoration of arginine, however, present the same difficulties here as in the question of growth. They are indefinite, confirming neither the positive results of Ackroyd and Hopkins nor the negative ones of Rose and Cook. If anything, they incline towards the latter, for the allantoin excretion appeared to have reached a minimum before the arginine was added to the diet. Still, the protocols of Rose and Cook show that in a long continued deprivation of arginine and histidine the fall in allantoin excretion was often irregular, and that such a halt was often followed by a further fall. Hence the present experiment can neither affirm nor deny that arginine has an effect on the purine excretion; one can only say that if such an effect exists, it is much less than that of histidine.

It must not be forgotten that in any case the conversion of histidine and arginine to purines (if such a conversion takes place) is only one of perhaps several possible metabolic paths. It may well be that in the case of histidine, which already contains the iminazole ring, this conversion proceeds rapidly in comparison with other competing reactions; whereas the reactions which convert arginine to a form suitable for purine synthesis have a velocity which is small compared with that of reactions which divert it into other metabolic paths (*e.g.* the formation of ornithine).

In considering whether these results justify the conclusion that histidine is a precursor of purines, it is of importance to note not merely the variations in the absolute amount of allantoin excreted when histidine is withdrawn from and restored to the diet, but also the variations of the ratio between the allantoin nitrogen and the total nitrogen of the urine. Should both allantoin and total nitrogen vary in the same sense and to the same degree (*i.e.* should the ratio of allantoin nitrogen to total nitrogen remain constant) it would appear probable that the alterations in the allantoin excretion were signs, not of any special disturbance of purine metabolism, but simply of nutritional failure generally. Now actually, while the nitrogen intake remained roughly constant, the total excretion of nitrogen in the urine by rats VII and VIII was 1·36, 1·40, 1·31, 1·28, 1·26, 1·34, 1·40, 1·39 and 1·43 g. in successive weeks. The slight decrease in the third, fourth, and fifth weeks, when the animals were receiving the diet free from histidine and arginine, is almost equal to the decrease in the allantoin excretion. In this experiment, as in that with rats V and VI, when arginine and histidine were withdrawn from the diet, the ratio of allantoin nitrogen to total urinary nitrogen showed a very considerable drop, corresponding to the fall in the absolute amount of allantoin excreted (Fig. 3). When histidine was restored to the diet the ratio again rose. With restoration of arginine alone, however, the ratio remained low, showing that any effect on purine metabolism possessed by this substance can only be slight. The evidence in favour of the conclusion that histidine bears a very intimate relationship to the purine metabolism is considerably strengthened by the fall in the allantoin excretion relative to the total nitrogen excretion when the two amino-acids are withdrawn from the diet and the subsequent rise when histidine is alone restored.

Summarising, one may say that the experiments completely confirm those of Ackroyd and Hopkins so far as histidine is concerned; that they do not support the suggestion that histidine and arginine are completely interchangeable in metabolism; and that they leave it doubtful whether arginine has any effect either in growth or in purine metabolism. The experiments of Ackroyd and Hopkins, confirmed here, and by Rose and Cook, afford ample evidence for concluding that histidine is, or may be, utilised by the growing animal for the manufacture of purines.

2. *The effect of arginine and histidine deficiency on adult rats.*

The experiments in which adult rats were used yielded a perfectly definite result. Rats I and II (Fig. 4) received the complete amino acid mixture through-

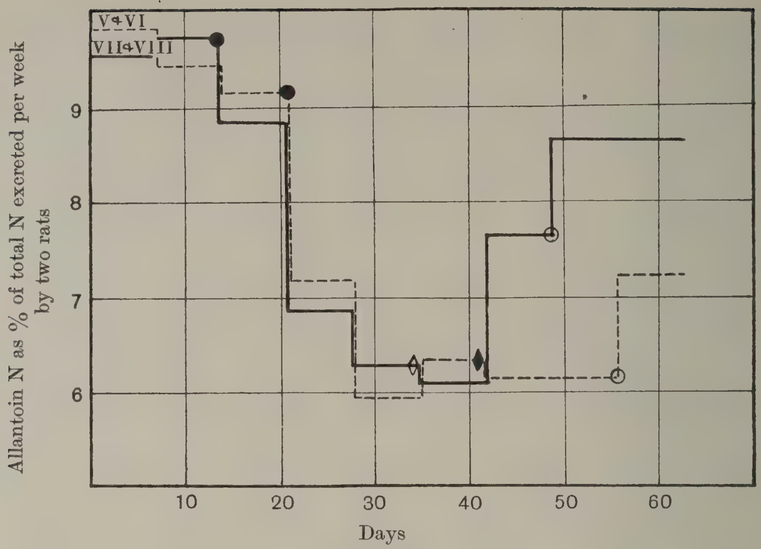

Fig. 3.

● Arginine and histidine removed from diet.

○ Arginine and histidine restored to diet.

◆ Arginine alone restored to diet.

◇ Histidine alone restored to diet.

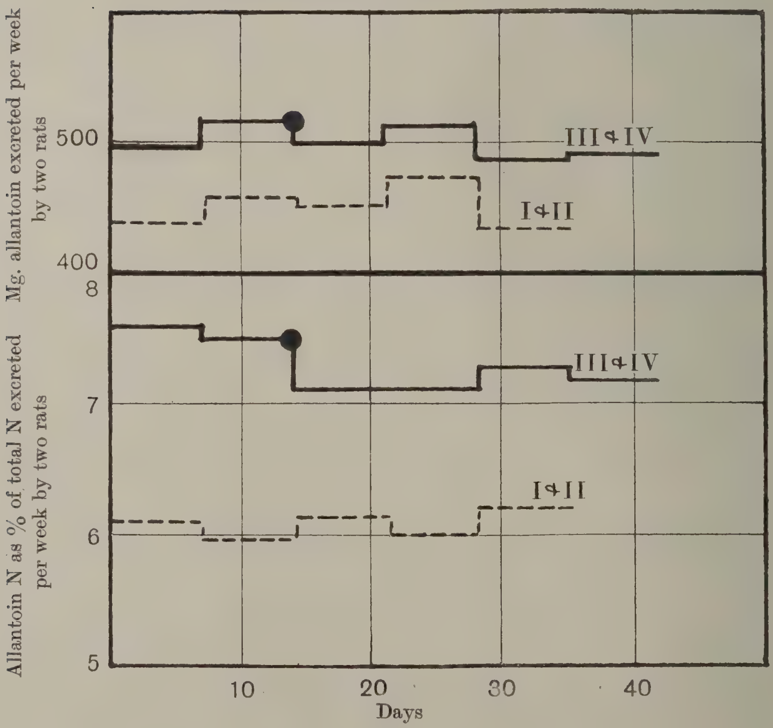

Fig. 4.

● Arginine and histidine removed from diet.

out the experimental period. Their weight remained constant, and so did both the amount of allantoin excreted per week and the ratio of allantoin nitrogen to total urinary nitrogen. Rats III and IV, from the same litter, gave similar constant results on a diet from which arginine and histidine had been withdrawn. Although the rats received the deficient diet only for 28 days, it seems quite certain that there is a very marked difference in histidine requirements between adult and growing animals, and the fact that the former are able to maintain themselves without external supplies of the amino acids readily explains the failure of such workers as employed them over similar periods to confirm the results of Ackroyd and Hopkins.

So far as body weight is concerned, Geiling [1917] claimed that addition of either arginine or histidine to a diet otherwise deficient rendered the nitrogenous portion of the diet adequate for maintenance of body weight in adult mice. The sole source of nitrogen in his deficient diet was that portion of a tryptic digest of caseinogen which was not precipitated by phosphotungstic acid, with the addition of cystine and tryptophan. Apart from the fact that, according to the data of Rose and Cox [1924], the protein was probably incompletely digested, the figures published by Geiling seem hardly able to support the interpretation put upon them.

Abderhalden [1922] has published the results of a series of feeding experiments, using adult rats, in which the nitrogen of the diet was derived entirely from a mixture of pure amino acids. He states that both arginine and histidine are indispensable amino acids. In one of his experiments addition of arginine did not remedy a deficiency caused by absence of histidine, so that his work does not support the idea of the interchangeability of the two substances. His curves certainly show that his animals lost weight when histidine and arginine were absent, but the multiplicity of the deficiencies renders his results exceedingly difficult of interpretation.

The experiments reported in the present paper do not, of course, necessarily mean that in the adult animal histidine no longer functions as a purine precursor. It may be that over the comparatively short period of the experiment the adult animal, though not the growing one, may be able to draw the necessary supplies of purine from some source as yet undiscovered; that histidine, whilst an important source of raw material in the young animal with its relatively greater need for purines, becomes a minor factor in the adult animal. In that case, just as readily as if histidine had no part whatever in purine metabolism, the adult, though normally utilising a certain amount of histidine to synthesise purines, would be able to subsist, at any rate for some time, without it. It is certain, however, that some difference exists in the need for histidine during, and after the cessation of growth.

The actual figures for the total nitrogen and the allantoin excretion show very clearly that in the young animal, not only is the nitrogen metabolism as a whole more active than in the adult, but that the purine metabolism is more active to an even greater degree. While all the animals were receiving the complete supply of amino acids, the young ones, in a week when their average weight was 61·5 g., excreted an average of 0·78 g. nitrogen and 0·209 g. allantoin per rat, while the allantoin nitrogen formed 9·55 % of the total. In the same week, the adults, with an average weight of 217·5 g. excreted 1·29 g. nitrogen and 0·243 g. allantoin per rat, the allantoin nitrogen being 6·7 % of the total. Thus the adults, more than three times the weight of the young ones, excreted only about 60 % more nitrogen, and only 16% more allantoin. In their case, too, the allantoin nitrogen formed a much smaller percentage of the total than in the case of the young ones.

SUMMARY.

On a diet deficient in histidine and arginine young rats lose weight, and their allantoin excretion, but not the total nitrogen excretion, falls almost to half the normal value. Restoration of histidine to the diet is followed by resumption of growth, and by increased allantoin excretion. It is concluded that, as suggested by Ackroyd and Hopkins, histidine functions as a precursor of purines in the animal body.

Restoration of arginine to the deficient diet is not followed either by appreciable growth or by increased allantoin excretion. The experiments, therefore, do not support the idea that arginine and histidine are completely interchangeable. Since, however, the restoration of arginine was followed by cessation both of the fall in weight and in allantoin excretion, it remains doubtful whether arginine plays any part at all in the purine metabolism.

Adult rats were able to live for 28 days without loss of weight or decrease in the allantoin excretion on a diet containing no more than traces of histidine and arginine.

The author is indebted to Prof. G. Barger, F.R.S., for the interest he has taken in this work, and for useful criticism and advice. The expenses of the research were partly defrayed by a grant from the Moray fund of this University.

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CLIX. THE MICROMETER SYRINGE.

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(*Received November 4th, 1925.*)

In the *Lancet* [Trevan, 1922] a piece of apparatus was described which could be used for the measurement of very small volumes of fluid, 0·01 cc. being measurable by its aid with an accuracy of less than ± 1 %.

In a slightly modified form the instrument has found considerable application, especially in the performance of the Ramon test for diphtheria antitoxin and in microchemical titrations, and it was thought that a description of the new form with some fuller details of the uses to which it has been put would be of interest. The principle of the instrument depends on the accuracy with which a fairly well-fitting piston of a glass syringe will displace a volume of fluid, the displacement being produced and measured by a Starret micrometer head such as may be obtained at any engineer's tool shop. Since the original paper was written, it has been discovered that a syringe driven by a screw had been used by both Kelvin and Lister, by the former for measuring relatively large volumes of gas in the constant pressure gas thermometer, by the latter for measuring small quantities of fluid in bacteriological research.

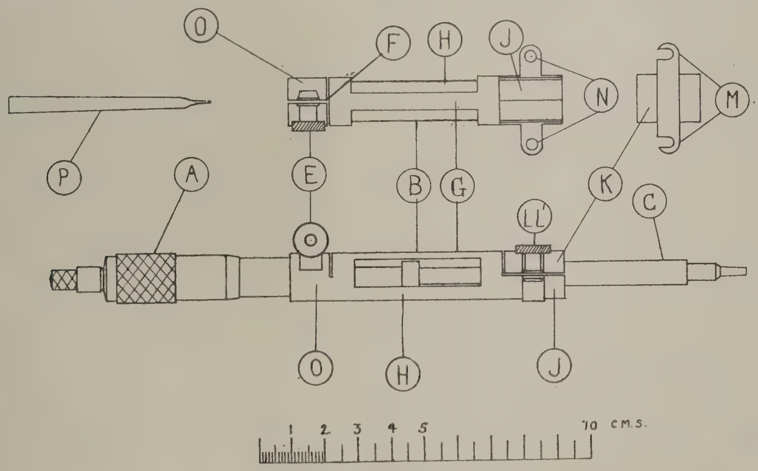

Fig. 1.

Fig. 1 shows the details. *A* is the micrometer head; *B* the holder, and *C* the syringe. The syringe illustrated is a glass "tuberculin" syringe with the piston oiled with viscous liquid paraffin, but any type of syringe can be used. The holder consists of a piece of steel tubing cut out to shape as illustrated. The end *O* is slotted as illustrated in the plan at *F* so that there is a slight give in the end sufficient for the micrometer head to be just held when it is pushed into this end of the holder. The milled head and screw *E* is provided to hold the micrometer head tightly in place. The tube is cut away leaving only the bars, *G* and *H*, the slots between allowing the head of the syringe piston to be drawn up and down. The syringe is held by the slip *JK*, the part *K* being removable. As shown in the plan, *K* is held down on *J* by the milled heads *LL′* which screw on to the sides of *J*. The lugs *M* on *K* are made with little hooks at the end which fit round the screws *N* so that *K* can be removed from *J* by merely slackening off *LL′*. *K* is lined with a piece of india-rubber pressure tubing attached with Chatterton compound, and *J* with two pieces of "fibre" arranged so as to form a "V" section groove for the syringe to lie in. The syringe is placed in the holder so that the head of the piston is just within reach when the syringe is emptied and fluid drawn up with the micrometer head withdrawn to its fullest extent. The syringe having been filled, the micrometer head is screwed down until contact is made with the piston when the instrument is ready for use. Using a micrometer head graduated in mm. in which one turn of the head corresponds to 0·5 mm. the average syringe ejects rather more than 0·01 cc. fluid for one turn. The distance through which it is necessary to move the piston to eject 0·01 cc. varies for the "tuberculin" type of syringe from 0·35 mm. to 0·55 mm., and readings of the divisions on the head can be estimated to one-tenth of a division or 0·001 mm. For some purposes it is best to choose a syringe which gives an even number of turns for 0·1 cc., *e.g.* 8, or best 10.

Calibration of syringes is best carried out by measuring the diameter of the piston of the syringe with a micrometer gauge and working out from the area of the piston the length the piston must traverse to displace 1·0 cc. Calibration by weighing mercury ejected gave figures such as the following:

Exp. 1. *Calibration with dry mercury. All-glass syringe.*

mm. reading on micrometer	Weight g.	Weight per 0·5 mm. g.
0·5	0·1267	0·1266
0·5	0·1282	0·1282
0·5	0·1272	0·1272
0·5	0·1268	0·1268
1·0	0·2545	0·1278
2·5	0·6440	0·1288
0·5	0·1270	0·1270
2·5	0·6330	0·1266
0·5	0·1270	0·1270
2·5	0·6405	0·1281
0·5	0·1270	0·1270
2·5	0·6385	0·1277
0·5	0·1285	0·1285
2·5	0·6390	0·1278
0·5	0·1265	0·1265
2·5	0·6337	0·1268
2·5	0·6395	0·1278

Mean 0·1276. Range ±0·9 %. Volume 0·00945 cc. = 0·5 mm.

The instrument can also be calibrated by titration. If it is filled with a strong solution of $CaCl_2$, small volumes can be ejected under the surface of water from a fine hypodermic needle and the chlorine ion titrated with $N/50$

$AgNO_3$. For the successful measurement of volumes of the order of 0·01 cc. it is best to eject the fluid under the surface of water to ensure accuracy in the separation of the last drop.

Exp. 2 *a. All-glass syringe.* 0·0143 cc. (= 0·5 mm. on micrometer) of a strong solution of $CaCl_2$. Titration figures; 4·42, 4·43, 4·44, 4·43, 4·44 cc. of $N/50$ $AgNO_3$. These figures do not correspond in absolute value to those in Exp. 2 *b* as different solutions of $CaCl_2$ and $AgNO_3$ were used. They are given to show the measure of agreement amongst the volumes ejected and not absolute values. It is clear that, as a good syringe does not leak back around the piston, if all the successive advancements of the piston are in agreement the absolute value must correspond to the value swept out by the advancing piston.

Exp. 2 *b.* Using 0·3 cc. syringe with metal piston with $CaCl_2$ solution. Titration figures; 6·35, 6·37, 6·38, 6·26, 6·22, 6·30, 6·27, 6·30, 6·30, 6·31 cc. $N/50$ $AgNO_3$.

Refilled syringe: 6·36, 6·40, 6·34, 6·28, 6·29, 6·34 for 2 mm.

Average 6·317 ± 0·055. Volume measured 0·004 cc.

The apparatus has been used for the measurement of 0·02 cc. of diphtheria toxin into a capillary tube for the performance of the Schick test and in the Ramon test by Glenny and Okell. A number of syringes are in daily use for this purpose by comparatively unskilled hands in these laboratories and are found to be simple and dependable.

Applications to microchemistry as a burette will be discussed at greater length in a further communication. Exp. 3 gives the results of titration of iodine with 0·096 cc. of $N/10$ thiosulphate.

Exp. 3. Approximately $N/50$ iodine. 5 cc. = 9·6 cc. $N/100$ thiosulphate, with an ordinary burette.

0·5 cc. iodine gave, in successive titration, the following figures: 0·0961, 0·0961, 0·0956, 0·0961, 0·0956, 0·0961, 0·0963, 0·0956, 0·0959, 0·0961, 0·0958, 0·0959, 0·0959, 0·0958, 0·0959 cc. as measured by the micrometer syringe using $N/10$ thiosulphate.

0·1 cc. of $N/100$ acid can be titrated using methyl red as an indicator with $N/50$ alkali with a discrepancy of at the most one in the third place. As Rehberg [1925] has pointed out, any further increase in the accuracy of micromethods of estimation can only be obtained by the use of smaller volumes. The reduction of the quantities required for titration to one-tenth of the present volumes used would be advantageous in many methods and such a reduction is certainly feasible with the apparatus described.

We think that this apparatus presents certain advantages over that of Rehberg in that the calibration of the bore of the syringe is a matter of a few seconds; there is no error of parallax, and the syringe holds sufficient fluid for many titrations or for titrations involving widely varying quantities of fluid. When titrating with acid the steel needle is replaced by a glass tube *P* drawn out to a point, and ground to fit the nozzle of the syringe. The point is covered on the outside with paraffin wax to prevent fluid running back. The final stages of the titration are carried out by allowing a small drop to collect at the tip and transferring the drop by a small glass rod (1 mm. in diameter) to the titrated fluid.

It has been used also as a gas pipette in a modified Haldane blood gas analysis apparatus. The piston is lubricated with liquid paraffin and gas leakage prevented by a layer of mercury on top of the piston, the syringe being used with the nozzle uppermost.

It can also be used as an accurate automatic pipette for large or small quantities of fluid. Exp. 4 gives a calibration by weighing dilute sulphuric acid showing the degree of agreement for a series of weighings.

Exp. 4. *Automatic pipette.* Weight of successive volumes of 1 cc. $N/10$ H_2SO_4: 1·000, 0·9999, 0·9994, 0·9994, 0·9994, 0·999, 0·9984, 1·000 g.

When used in this manner the micrometer head is screwed down until the piston rests on the plug at the bottom of the syringe, the reading on the micrometer head is then taken and the micrometer head is screwed back an amount corresponding to the volume required. Fluid is then drawn into the syringe until the head of the piston meets the micrometer plunger, and the fluid is then ejected by allowing the piston of the syringe to fall until the syringe is emptied. By joining a rubber band to the head of the syringe and passing it round the milled head E the syringe can be used for abstracting known volumes of blood slowly from a vein, the rubber band drawing the syringe piston slowly upwards as the micrometer is screwed out. When used as an automatic pipette, larger syringes of any volume required can be fitted into similar holders.

It may be pointed out in passing that a good hypodermic syringe may be used for the measurement of volumes without any attachment, with as much accuracy as a pipette of the same volume. For example, the piston of a tuberculin syringe can be set by eye with an error of less than ± 0·1 mm., which corresponds to 0·002 to 0·003 cc., and there is no error of parallax or of drainage as with a graduated pipette. We have also used large syringes with success for the collection of gas samples for analysis, displacing the usual cumbersome mercury bulbs, lubrication of the piston with a little water being sufficient to prevent leakage. We have also used them in place of separation funnels for small quantities of blood.

I take this opportunity of thanking Miss Boock, B.Sc., and Miss Attwood for performing many measurements for checking the accuracy of the instrument, and my mechanic, F. Gowlett, for his care and skill in making various experimental models.

Any metal worker can construct the instrument from bicycle frame tubing, but for those without facilities Messrs Burroughs Wellcome & Co., Snow Hill, London, E.C., have kindly arranged to supply the instrument with a calibrated syringe of a suitable bore.

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CLX. STUDIES ON THE PHYSIOLOGY OF PLAIN MUSCLE.

IV. THE LACTIC ACID CONTENT OF PLAIN MUSCLE UNDER VARIOUS CONDITIONS.

By CHARLES LOVATT EVANS.

From the Harvey Physiological Laboratories, St Bartholomew's Medical College, London.

(*Received November 8th, 1925.*)

Apart from the very general belief that most tissues may readily be made to yield lactic acid, there is very little to indicate the presence of that substance in plain muscle. It is true that Vincent and Lewis [1901] found, as Lehmann had done before, that the normally alkaline reaction of the tissue tended towards neutrality or acidity when the tissue was kept, and further that some of the older observers, employing the rough tests which were then available, claimed to have demonstrated the presence of lactic acid in plain muscle. Yet there is a remarkable contrast between the intimate knowledge which has been yielded by the application of modern methods to the study of the metabolism of striated muscle, and the almost complete ignorance which prevails with regard to the chemical properties of plain muscle. The pivotal importance of lactic acid in the physiology of striated muscle induced me to study the relation, if any, which this substance might bear to the physiology of plain muscle.

Presence of Lactic Acid in Plain Muscle.

Various methods have been employed for the detection and estimation of lactic acid. When this work was commenced six years ago, large amounts (from 20 to 700 g.) of tissue were worked up by Fletcher and Hopkins' [1907] method as used for frog's striated muscle. In using the larger amounts there was no doubt some loss, but it was certain that the amount of lactic acid present was small compared with that in striated muscle. Thus, from 700 g. of muscle from the horse stomach, which had been minced and slowly heated to boiling, I obtained an amount of crystalline zinc salt corresponding to only 0·043 g. lactic acid. In other experiments with the zinc method, using muscle from the small intestine of the dog, and the ox retractor penis, the lactic acid content of the fresh tissue was found to vary from 0·012–0·076 %. It would serve no useful purpose to give details of these earlier and more imperfect experiments; but it was possible, from them, to state that lactic acid did occur, though only in small amount.

Analysis of the crystalline zinc salt obtained in some of these experiments and dried over calcium chloride gave the following results:

Exp.	% water of crystallisation (theory requires 12·9 %)	% ZnO in dry salt on incineration (theory requires 33·4 %)
1	13·3	35·1
2	11·8	—
3	12·7	37·8
	Mean = 12·6	Mean = 36·5

The salt was obviously not quite pure.

Specimens of the salt gave Hopkins' thiophene test: when distilled with 0·25 % sulphuric acid and a little manganese dioxide, acetaldehyde was formed, which was recognised by its smell, as well as by Schiff's test, the mirror test, and the iodoform reaction. An attempt to determine the optical rotation of the free acid was unsuccessful because too little of it was obtained. I have nevertheless no hesitation in saying that sarcolactic acid is recoverable in small amounts from plain muscle from various sources.

Methods.

In addition to the method used by Fletcher and Hopkins, I have also tried the extraction method described by Wolf [1914] and the various procedures recommended by Meyerhof [1920, 1; 1921], but finally have found that, with slight alterations, the extraction method of Hirsch-Kauffmann [1924] together with the lactic acid estimation as described by Clausen [1922] gives the most regular and accurate results. The outlines of the treatment of material for resting controls are as follows: from 3–6 g. of the tissue is cooled in a beaker surrounded by a mixture of ice and salt, and weighed. The tissue is placed in a nickel crucible, which is wrapped in a duster; liquid carbon dioxide is then blown into the crucible, which is quickly filled with solid CO_2, in which the tissue is frozen. While thawing it is cut up with scissors and dropped into 10 cc. of ice-cold 4 % HCl saturated with NaCl; about 1 g. of white sand is added and the whole ground up in a mortar; 10 cc. of 5 % mercuric chloride is then added, and the mixture left overnight. It is then filtered through a dry paper into a graduated cylinder, the residue being wrung out in muslin. The volume of the filtrate is noted, and it is then carried through the process exactly as described by Hirsch-Kauffmann, removal of carbohydrate being effected by 2 cc. of 10 % $CuSO_4$ and 5 cc. of 25 % milk of lime. With the quantities stated, the amount of lactic acid in the final filtrate will be about half that present in the first mixture of tissue and reagents: the loss from adherence to precipitates and filter papers can, of course, be calculated from the ratios of the volumes of the materials at each stage of the process.

To the final filtrate used for the lactic acid estimation enough dilute sulphuric acid is added to make 0·5 % concentration. Oxidation by permanganate and the subsequent titration of the aldehyde bisulphite compound are carried out exactly as described by Clausen, and need not be further described. For tissues subjected to various preliminary treatments, slight and obvious modifications of the initial stages are introduced. Thus when treated with fluids, the fluid was added to the initial mixture and its volume allowed for.

The tissues employed were the following: of mammals, the intestine, uterus, oesophagus and bladder of the cat, the intestine of the dog, and the retractor penis muscle of the bullock; of cold-blooded animals the stomachs of the frog and tortoise (*Testudo graeca*). The tortoise stomach proved best of all, because of the regularity of the resting controls, due to the fact that lactic acid did not accumulate very rapidly after removal of the tissue from the body. The tissue also shows no spontaneous contractions. When an experiment on one portion of tissue was contemplated, the stomach was removed, opened, and the mucosa stripped off from the intestinal towards the oesophageal end: the tissue which remains weighs about 4–6 g. and has been shown by histological examination to consist almost entirely of plain muscle, the fibres of which run in a roughly circular direction, as in the frog. The band of tissue is divided longitudinally into two portions, one of which serves as the control, and the other for the experiment. When it was desired to carry out a number of experiments against a single control, the tissue from several stomachs was prepared, cut into pieces about 1 cm. square which were thoroughly shuffled, and samples of 3–6 g. used for control and experiments. The variation of the resting lactic acid value in different stomachs is as a rule so small that no appreciable error is introduced by this method of cutting and shuffling.

Mammalian tissues are much more difficult to employ; there is a rapid post-mortem accumulation of lactic acid and there is the further difficulty that in most cases the tissue exhibits spontaneous rhythmic contractions, whilst for various reasons the difficulty of obtaining representative samples for controls is much greater. In the small intestine, for instance, the resting lactic acid values depend on the degree of spontaneous activity, which as pointed out by Alvarez [1922] shows a diminishing gradient from above downwards. I have already shown [1923] that the oxygen usage of intestinal muscle shows a similar gradient, so that I was not surprised to discover the following resting lactic acid values, estimated by Fletcher and Hopkins' method, for the plain muscle from different levels of the dog's intestine:

Level	Exp. 4 %	Exp. 5 %	Exp. 6 %
1 (highest)	0·062	0·06	0·076
2	0·046	—	—
3	0·036	0·047	0·039
4 (lowest)	0·024	—	—

In order, therefore, to carry out a satisfactory experiment on intestinal muscle, it is necessary to select a portion of the gut and to divide the muscle lengthwise, using one part as a control and the other part for the experiment; a cutting and shuffling method would, I think, be unsafe, so that a large series of experiments against one control could not easily be carried out.

"*Resting*" *values of lactic acid.*

From none of the tissues examined was lactic acid entirely absent, even when the tissues were in the freshest condition. The value is always low however, particularly in the plain muscle of cold-blooded animals, but not so low as in long-rested skeletal muscle. The following table gives some typical results.

Table I. *Resting values of lactic acid.*

Tissue	Lactic acid %			
Small intestine of dog	0·029	0·046		mean = 0·042
	0·051	0·036		
	0·012	0·024		
	0·062	0·060		
	0·047	0·076		
	0·039	0·031		
	0·038	—		
Intestine of cat	0·178			
Retractor penis of bullock	0·076	0·072		mean = 0·139
	—	0·27		
Cat uterus	0·141	0·024		mean = ·083
Cat oesophagus	0·05			
Cat bladder	0·214			
Frog stomach	0·03			
Tortoise stomach	0·056	0·029	0·023	mean = 0·027
	0·013	0·013	0·026	
	0·032	0·029	0·049	
	0·014	0·016	0·020	

Methods of fixation of the tissue. It is very important to be able to kill the tissue and to fix the lactic acid precursor, whatever it may be, in such a manner as will lead to the minimal production of lactic acid during the killing. In the case of frog's striated muscle, cooling in liquid air or even in ice and salt is no doubt adequate, but it does not necessarily follow that this method will yield the best results with plain muscle, which often shows strong contractions on cooling. I have therefore compared the cooling method, using liquid carbon dioxide, with killing by crushing in ice-cold 4 % HCl, and with killing by dropping into boiling 4 % HCl, with the following results:

	Lactic acid %	
	(1)	(2)
Fixed in boiling 4 % HCl	0·014	0·026
Control frozen and fixed in cold 4 % HCl ...	0·016	0·023
Fixed in ice-cold 4 % HCl, and at once ground up	0·02	—

This experiment shows that the three methods give practically identical results; the boiling method is less satisfactory in one respect, namely, that some hydrolysis of protein occurs, so that, after treatment with mercuric chloride, there is a tendency for the liquid to froth during the subsequent passage of H_2S and air, and the cold methods seem to be preferable on that account.

The effect of stimulation on the lactic acid content.

For this purpose the tissue was suspended in a moist chamber containing air or oxygen and stimulated for 1–2 seconds at intervals of 20 or 30 seconds with a galvanic current of sufficient strength to cause the maximum contractions, these being recorded by a lever. The current required varied from 5–20 milliamperes. This stimulation was repeated, by means of a rotating key, until the muscle showed definite signs of fatigue. Meanwhile, the control sample of tissue was suspended in a similar moist chamber and both samples were fixed simultaneously.

Table II. *Effect of repeated stimulation.*

Tissue	Stimulation	Lactic acid %
12 frog stomachs (20°)	0 (control)	0·03
	5 ma. at 45″ intervals for 2 hrs.	0·04
Cat bladder (38°)	0 (control)	0·214
	15 ma. at 20″ intervals for 1 hr.	0·241
Tortoise stomach (20°)	0 (control)	0·029
	5 ma. at 60″ intervals for 1 hr.	0·085
Tortoise stomach (20°)	(Control)	0·013
	25 ma. at 20″ intervals for $2\frac{1}{4}$ hrs.	0·068
	25 ma. at 20″ intervals for $3\frac{1}{2}$ hrs.	0·086
Frog striated muscle (20°)	(Control. Not quite resting)	0·10
	45 mins. tetanus	0·34

The experiments show that there is an undoubted production of lactic acid during contraction, but the amounts so formed are small when compared with those set free by striated muscle. This must be either because the recovery process is abnormally rapid, or because the liberation of lactic acid comes early to a standstill, or else because the content of lactic acid precursor is lower in plain than in striated muscle. As we shall see, the last reason is an important one, but the second one probably also holds good.

The effect on plain muscle of conditions which in striated muscle cause rigor mortis.

Table III.

Tissue	Treatment	Lactic acid %	
(1) Dog intestine	Gradually heated to 70°	0·083	
	Control	0·029	
(2) Dog intestine	30 mins. in $CHCl_3$-Ringer	0·032	0·069
	Control	0·012	0·047
(3) Bullock retractor	Gradual heat to 80°	0·144	
	Control	0·076	
	Sudden heat to 80°	0·079	
(4) Bullock retractor	4 hrs. $CHCl_3$-Ringer	0·32	
	Control	0·27	
(5) Cat uterus	$CHCl_3$ vapour 2 hrs.	0·14	
	Control	0·14	
(6) Tortoise stomach	Gradual heat to 60°	0·078	
	Control	0·056	
(7) Tortoise stomach	30 mins. in $M/15$ Na_2HPO_4 plus 0·4 % caffeine	0·043	
	30 mins. in $M/15$ Na_2HPO_4	0·059	
	Resting control	0·026	
(8) Frog skeletal muscle (May)	$CHCl_3$ vapour for 2 hrs.	0·182	

This table shows that, with the exception of series 5 and 7, treatment, which applied to skeletal muscle would lead to the onset of *rigor*, causes also in plain muscle a rise in the lactic acid content. The maximum for plain muscle is, however, usually lower than that which would be expected for striated muscle. Perhaps this is partly due to the less efficient buffering power of plain muscle, but the store of lactic acid precursor being smaller also limits the amount of acid formed.

In series 5, exposure to chloroform vapour produces no alteration in the lactic acid content; this is perhaps due to the fact that the control already shows the lactic acid maximum.

In series 7 it is seen that, although there is an increase in the lactic acid after 30 minutes' immersion of the minced tissue in a phosphate solution containing 0·4 % of caffeine, this increase is much smaller than that found on immersion in the phosphate alone; the caffeine appears to have inhibited the production of lactic acid. Although it would be unsafe to draw a conclusion from the result of a single experiment, this fact, if confirmed by subsequent study, might perhaps be related to the further fact that whereas caffeine exerts a stimulating action on striated muscle, its effect on plain muscle seems to be more usually to cause relaxation. This question, together with the further question of the relation of lactic acid production to contraction must be deferred for the present.

The effect of mincing in buffered solutions.

The rate of formation of lactic acid in tissue which has been minced in buffer solutions of different hydrogen ion concentrations was studied in a manner similar to that used for striated muscle by Laquer [1914] and Meyerhof [1921]. The tissue, 3–6 g., was weighed, placed in 5 cc. of the buffer solution, minced therein with scissors, left for the desired time, and then killed by the addition of 10 cc. of ice-cold 4 % HCl followed by immediate disintegration with sand in a mortar.

Table IV. *Immersion of minced tissue in buffer solutions.*

Tortoise stomach muscle (18·5°).

	% lactic acid formed in				
Duration of exp.	$M/15$ NaH_2PO_4 (p_H 4·5)	$M/15$ Na_2HPO_4 (p_H 9·0)	$M/15$ Na_2HPO_4 plus 0·30 % Na_3AsO_4	0·8 % $NaHCO_3$ (p_H 9·5)	Control
15 mins.	—	0·043	0·047	—	0·026
30 ,,	—	0·059	0·061	0·051	0·026
1·25 hrs.	—	0·081	0·086	—	0·023
2·5 ,,	—	0·078	—	0·087	0·023
3·25 ,,	0·054	—	—	—	0·024
3·25 ,,	—	0·089	—	—	0·032
5·75 ,,	0·058	—	—	—	0·024
5·75 ,,	—	0·087	—	—	0·032
19·5 ,,	0·162	0·165	—	—	None
24 ,,	—	—	0·13	—	0·026

In some of the experiments 0·3 % of sodium arsenate was added to the disodium phosphate solution to compare the results with those obtained under similar conditions by Meyerhof with striated muscle. The results are shown in Table IV and are plotted in Fig. 1.

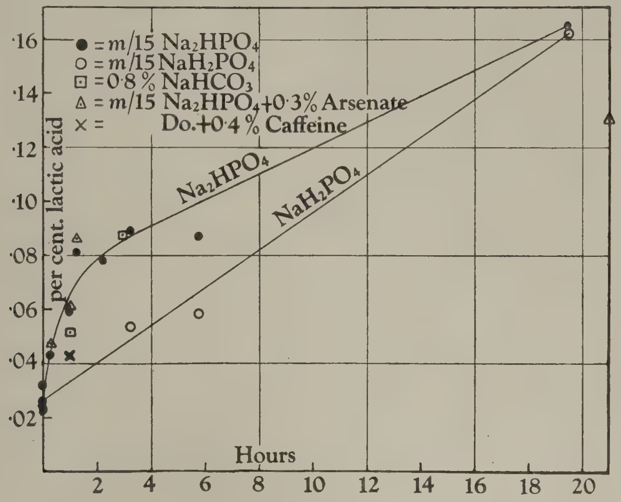

Fig. 1.

The experiment shows that, as in striated muscle, there is a rapid accumulation of lactic acid in the disodium phosphate solution, and a still more rapid one in the alkaline arsenate solution; the amount finally formed (in 20 hours) in tortoise stomach muscle would seem to be about 0·13–0·165 %, and it would appear that this represents complete conversion of all the precursor into lactic acid; at all events, I have not been able to exceed that amount by any treatment of the tortoise stomach muscle.

In the acid phosphate solution, on the other hand, owing to the acid reaction, the rate of lactic acid formation is much slower, in which again there is close resemblance to what happens in skeletal muscle; but bicarbonate solutions do not seem to differ sensibly from the alkaline phosphate solution. This may be either because the amount of tissue was great when compared with the volume of the bicarbonate solution in which it was suspended, so that the phosphate concentration was still adequate, or because the phosphates do not play such an important part in the conversion of lactacidogen into lactic acid in plain as in skeletal muscle. The point must be decided by further experiments.

Comparative experiments made with the striated muscle of the tortoise showed that it behaves in a similar manner to that of the frog under comparable conditions. Although the lactic acid maximum attained with plain muscle is much below that for striated muscle, it would appear that the relative rate of development is at least as great, if not greater, in plain muscle; thus in disodium hydrogen phosphate with or without arsenate, more than half the maximal yield is obtained within an hour, the increase over the resting value being about 200 % in the arsenate solution, though only about 50 % in monosodium dihydrogen phosphate solution.

Lactic acid formation in anaerobiosis and in survival in oxygen.

So much light has been thrown on the chemical processes in striated muscle by the study of the effects of anaerobiosis, that the results of similar experiments on plain muscle cannot fail to be instructive. Experiments were therefore carried out to compare the lactic acid content of the tissue after various periods of aerobic and anaerobic survival; anaerobiosis was effected by immersing the tissue in frog Ringer solution containing $N/1000$ NaCN, or by exposing the tissue without immersion in fluid to a continuous current of moist commercial nitrogen; in the one case comparison was made with tissue suspended in oxygenated Ringer, and in the other case with tissue placed under similar conditions in a stream of commercial oxygen. In all cases the tissue was used in pieces about 1 cm. square; undue injury due to mincing was thus obviated, but the procedure had the drawback that in spite of the thinness of the pieces of tissue, the oxygen supply during the aerobic periods could not have been completely efficient; the tissue suspended in oxygenated Ringer was agitated by shaking at frequent intervals in an atmosphere of oxygen, but in the experiments in which the tissue was exposed directly to the moist gas, the latter could usually only have access to one surface of each piece of tissue. Therefore the aerobic periods were in reality periods in which a supply of oxygen was offered. The results indicate that the access of oxygen was slightly behind the requirement, so that there was some accumulation of lactic acid; this was also the case in most of the experiments of Meyerhof on striated muscle.

The results of these survival experiments are given in Figs. 2 and 3. These graphic results speak for themselves; during anaerobiosis there is a rapid accumulation of lactic acid in the resting tissue, whilst during aerobic survival the lactic acid mounts up very slowly indeed. It would seem that the lactic acid maximum is almost reached at the end of about 8 hours' survival at room temperature; when the asphyxiated muscle is returned to oxygen, the lactic acid begins to disappear again; this disappearance occurs even after the tissue has been in nitrogen for 20 hours, as the ensuing 7 hours in oxygen show. It would seem that with protracted survival, the efficiency of recovery gets less and less, so that after 20 hours in nitrogen followed by 24 hours in oxygen (not shown in the figure), there is more lactic acid present than there was after the tissue had been in nitrogen for 20 hours and in oxygen for 7. Nevertheless, in spite of this, there was less lactic acid after the survival of 44 hours, the first 20 of which were in nitrogen, than there was after a survival of only 6½ hours all of which was spent in nitrogen.

The maintained low resting level of lactic acid when the tissue is kept in oxygen, together with the demonstrated removal of lactic acid when the anaerobically surviving tissue is restored to oxygen, provide incontrovertible evidence that, as in striated muscle, there is a continuous removal, by oxidative means, of the lactic acid which is being formed continuously as a result of metabolic processes in the tissue. There is, then, in oxygen, some sort of a recovery process, and the next step is to enquire whether or not this is of the same nature as that which occurs in striated muscle. There are three ways by which this problem may conveniently be approached, namely, by the study of the thermal aspects, from the oxygen consumption, and from the carbohydrate content. I have carried out attempts along the last two directions.

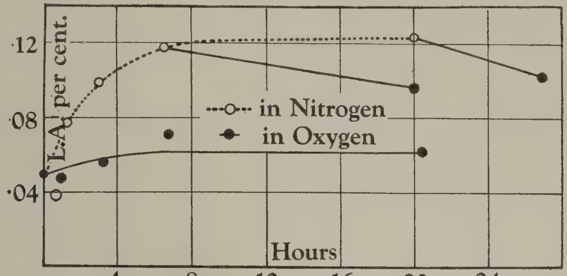

Fig. 2.

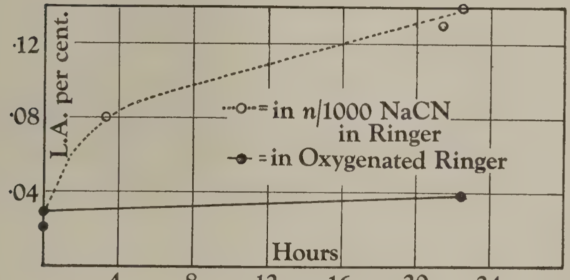

Fig. 3.

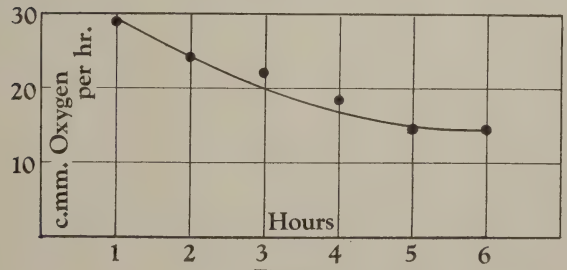

Fig. 4.

The oxygen requirement of resting plain muscle.

The oxygen requirement of resting plain muscle was determined by the use of the microrespirometer, a sensitive instrument of the Krogh pattern, bottles of about 10 cc. capacity being used. Thin strips of tissue from the experiment of Fig. 2 were cut with a sharp razor, and floated in 1 cc. of Ringer's solution: the apparatus was shaken horizontally, with the bottles immersed in a water-bath at room temperature (17°). The rate of oxygen usage was determined each hour for 6 hours, and the results per g. per hour are given in Fig. 4. The results were high during the first half-hour and were rejected; this high value is probably due to oxidative removal of lactic acid formed in the process of preparation involving handling and oxygen lack. In the succeeding hours the oxygen usage also showed a slow decline towards a steady value of about 15 mm.^{3} per g. per hour. I have shown in a previous paper [1923] that after a period of oxygen lack there is an accelerated rate of oxygen usage, and the evidence which has now been put forward indicates that this is associated with oxidative removal of lactic acid, much as happens in striated muscle.

It is easy to calculate, as Meyerhof has done for striated muscle, to what extent the oxygen utilisation corresponds to the lactic acid reduction. Each milligram of lactic acid which is oxidised necessitates the utilisation of 744 mm.^{3} of oxygen. In the experiment shown in Fig. 2 there has been in nitrogen an accumulation of lactic acid which has raised the percentage in 6 hours from 0·049–0·116 %, or an increase of 0·67 mg. per g. of tissue, whilst in oxygen the increase in the same time is only 0·12 mg. per g.; thus, the presentation of oxygen has in 6 hours prevented the formation of 0·55 mg. lactic acid. (If there had been complete lack of oxygen from the very beginning (*i.e.* no free oxygen present in the tissue), even more lactic acid would have accumulated.)

This amount of lactic acid would require 410 mm.^{3} of oxygen to oxidise it, and, if the lactic acid were removed entirely by oxidation, the tissue should use 410 mm.^{3} oxygen in 6 hours; but adding up the oxygen usage from the experiment shown in Fig. 4, we find that actually only 123 mm.^{3} of oxygen were used in that time. That is to say that only about one-third of the lactic acid is oxidised (actually 1/3·35), the remainder being removed in some other form. This so closely resembles what happens in skeletal muscle, that, although I have no final proof for it, it seems almost certain that what has happened to the remaining two-thirds of the lactic acid is a restoration to the lactic acid precursor, presumably glycogen.

Relation of carbohydrate to the origin of lactic acid.

For the investigation of this question I have made some determinations of the glycogen content, and of the total hydrolysable carbohydrate content of plain muscle under various conditions, but with results which are at present puzzling, and which require further examination. Glycogen has been estimated by a micro-adaptation of Pflüger's method, the glycogen thrown down by alcohol being collected by centrifugation, and being finally hydrolysed by 2 % HCl at 100° for 3 hours: determination of the glucose so produced was carried out by the Shaffer-Hartmann method. Hydrolysable carbohydrate was determined by initial hydrolysis of the fixed tissue by 2 % HCl, deproteinisation by $HgCl_2$ followed by removal of mercury by H_2S, neutralisation, and determination by Shaffer-Hartmann. In other cases extraction has been carried out by cold alcohol, glycogen estimated in the residue, and soluble carbohydrate in the filtrate after deproteinisation by colloidal ferric hydroxide [cf. Meyerhof, 1920, 2]. Some results are given in Table V.

Table V. *Carbohydrate content of plain muscle.*

Tissue	State	Glycogen %	Soluble carbo-hydrate %	Total carbo-hydrate %	Lactic acid %
Retractor	Fresh	0·012	—	—	0·072
	Rigor	0·022	—	—	0·079
Cat uterus	$CHCl_3$	None	—	—	0·141
Intestine (dog)	Fresh	0·005	—	—	0·178
Retractor	Fresh	0·018	0·061	—	0·27
	Fresh	0·032	0·055	—	0·22
	$CHCl_3$	0·014	0·062	—	0·32
Tortoise stomach	Fresh	0·05	—	0·51	0·02
	Cyanide (21 hrs.)	0·014	—	0·495	0·13

There are three striking features about these estimations; (1) the low value of the glycogen, (2) the relatively high value of the remaining carbohydrates, especially in the tortoise tissue, (3) the fact that where there is a comparison between the lactic acid and glycogen of resting and treated tissues, the increase in the lactic acid is many times greater than the decrease in the glycogen content. Three explanations suggest themselves: (1) that the lactic acid does not arise from carbohydrate at all, (2) that it arises from some carbohydrate other than glycogen, but that the results of the total carbohydrate determinations are spoilt by the production of carbohydrate from some other source in the course of hydrolysis, (3) that the glycogen estimations are unreliable. I incline to the last-mentioned opinion, for reasons now to be stated.

Similar experiments carried out on frog's striated muscle gave satisfactory results: for these, I used 1 or 2 g. of muscle. Expecting, from the lactic acid maximum, that the glycogen content of plain muscle would be of the order of 0·1–0·2 %, I therefore used 5 or 6 g., or in some cases 20 g. of tissue, but the results were always low. Now the technique used was in both cases the same, namely, for each g. of tissue there was used 1 cc. of 60 % KOH for hydrolysis, then 2 cc. water were added, and 4 cc. alcohol: in the reprecipitations 2 cc. water and 4 cc. alcohol were used; the volume of fluid from which precipitation took place would thus be disproportionately great when the glycogen content of the tissue was small, and the relative loss would tell very seriously on the result.

In order to test this explanation, I have made the following simple experiment: frog's striated muscle was minced at – 5°, and was divided into samples of various weights; in two of these the glycogen was estimated in the usual way, but in others the alcohol precipitation was carried out with much larger volumes of fluid. The results are shown below:

Exp.	Weight of tissue g.	Volume of fluid from which glycogen was precipitated (cc. per g. tissue)	Minimal weight of glycogen present (calculated from Exp. 1)	Volume of fluid per mg. minimal glycogen content cc.	Yield of glycogen %	Apparent glycogen content of tissue %
1	1·8	8·3	14·7	1·06	100	0·82
2	0·46	13·0	3·75	1·6	62	0·50
3	0·44	34·0	3·6	4·3	23	0·19
4	0·26	58·0	2·13	7·3	14	0·12

It is evident from these results that the greater the volume of dilute alcohol from which the glycogen is thrown down, the lower the results obtained.

This is also shown by the following results of experiments in which equally small amounts of glycogen were estimated, but in which the volume of precipitating fluids was kept as small as possible throughout:

Exp.	Wt. of tissue g.	Vol. of fluids per mg. minimal glycogen content cc.	Yield of glycogen, calc. from Exp. 1 (%)	Apparent % glycogen in tissue
1	1·73	0·32	100	0·88
2	0·63	0·31	103	0·91
3	0·32	0·47	98	0·86

Hence, in carrying out the estimation of such small amounts of glycogen as must be present in plain muscle, the volume of alcohol should be adjusted, not to the weight of the tissue taken, but to the weight of glycogen likely to be present. Experiments of this nature are now in hand.

In this lack of correspondence between the glycogen content and lactic acid maximum, as well as in some other respects, plain muscle appears to bear a strong chemical resemblance to cardiac muscle, which has recently been investigated by Katz and Long [1925] and by Hines, Katz and Long [1925].

SUMMARY.

1. Lactic acid is present in small amount in "resting" plain muscle. (0·03–0·08 %; the larger amounts in mammalian tissue.)

2. The amount of lactic acid is increased when the muscle is made to contract. The lactic acid maximum is never so high as in skeletal muscle from the same species.

3. The amount of lactic acid is increased by treatment which in skeletal muscle produces *rigor*. The highest amount thus found was 0·32 %, and usually it was below 0·2 %.

4. When placed in solutions of phosphates, the lactic acid production is much greater when the p_H of the solution is 9, than when it is 4·5. The rate of lactic acid formation in disodium hydrogen phosphate solutions is accelerated by the presence of arsenates. The rate of acid production, under the conditions of experiment, was much the same in 0·8 % $NaHCO_3$ as in disodium hydrogen phosphate solution.

5. The lactacidogen content for the muscle of the tortoise stomach (Autumn) is of an order corresponding to about 0·15 % lactic acid.

6. Lactic acid rapidly accumulates in plain muscle when this is kept under anaerobic conditions, but scarcely at all when kept in oxygen.

7. The oxygen usage of resting plain muscle indicates that the recovery process in oxygen is of much the same nature as that in skeletal muscle; actually the fraction oxidised under experimental conditions was about one-third.

8. Owing, it is thought, to the errors incidental to the determination of small amounts of glycogen, it has not been possible, up to the present, to demonstrate that lactic acid arises from glycogen, or indeed, from any carbohydrate. In any case the glycogen content of the tissue is small, though, when allowance is made for the possible errors of experiment, perhaps large enough to make it possible that glycogen is the parent substance from which lactic acid is formed.

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CLXI. THE INTERACTION OF FREE AMINO-NITROGEN AND GLUCOSE.

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In the course of an investigation into the influence of added glucose upon the action of tissue proteases, an interaction of glucose with the free amino-group was observed. In alkaline solution in the presence of this sugar, the free amino-nitrogen of glycine and of other more complex derivatives of protein, such as a peptic digest of albumin, Witte's peptone, and Parke-Davis' peptone, was found to decrease. Three preparations of glycine, two commercial and one obtained in this laboratory, when allowed to react separately with each of four specimens of glucose from different sources, gave in each case qualitatively and quantitatively similar results. The reaction was found to proceed unchecked in solutions of tested sterility and to be unaffected by preliminary boiling or autoclaving of the reagents.

When a peptic digest in which the above reaction had occurred was submitted to the method of fractional analysis for incomplete protein hydrolysates devised by the authors [1925], the reduction in free amino-nitrogen was found to have been unaccompanied by a synthesis of the lower fragments into more complex derivatives; nor was any loss in total nitrogen, formation of ammonia, urea or cyanic acid discovered even, in the case of glycine, with an 85 % diminution of the free amino-nitrogen.

Boiling in weak acid solutions when phosphate is present restores but slightly the free amino-nitrogen which has disappeared during the incubation with glucose; in the absence of phosphate this procedure restores in large part the lost free amino-nitrogen.

On the other hand, the incubated solution of protein derivative and glucose was found to have acquired the property of reducing methylene blue in neutral or even slightly acid solutions in the absence of any added enzyme. This reduction was usually carried out at 56°, to ensure sterility, but it also occurs, more slowly, at 37°. With glycine and peptone, and probably therefore with other protein derivatives, the reduction of methylene blue is greatly accelerated by the presence of phosphate. Unhydrolysed albumin after incubation with the sugar rapidly reduced methylene blue without any added phosphate. The addition of washed muscle to an incubated mixture of peptone and glucose accelerated the reduction of methylene blue.

Acetamide, urea, or creatine added to glucose did not reduce methylene blue at a lower p_H or at a greater rate than the sugar alone.

It has not been found possible as yet to complete an investigation of the nature of the products occurring in the preliminary incubation and the subsequent oxidation. In the meantime, it was deemed of value to publish the results of a preliminary qualitative survey of the facts discovered, in view of the recently published observations of Raper and Wormall [1925], Happold and Raper [1925], Robinson and McCance [1925] and Onslow and Robinson [1925] on the induction of oxidative deamination of amino-acids by certain phenols, and of the discovery of a new oxidation-reduction system composed of glycine, acetaldehyde and phosphate by Haehn and Pülz [1925].

The increased oxidation of protein and amino-acids by alkaline permanganate in the presence of glucose has been observed by Fosse [1921] and also in the presence of formaldehyde by Fearon and Montgomery [1924]. The latter workers suggested, in explanation of the effect of formaldehyde, a destabilisation of the amino-group by its preliminary combination with the aldehyde group.

In incubated solutions of glucose and glycine, of which details are given below, the sugar content, analysed by copper reduction in alkaline solution, showed no change.

The accelerating influence of phosphate on the oxidation of glucose by H_2O_2 has been studied by Löb and his collaborators [1910, 1911, 1912; Beysell and Löb, 1915] and by Witzemann [1920]. Harden and Henley [1922] showed that phosphate, though accelerating the oxidation of glucose by H_2O_2, was not indispensable. The influence of phosphate on the reduction of methylene blue by glucose and glycine or peptone, described above, is directly analogous.

Warburg and Yabusoe [1924] observed the oxidation of fructose by atmospheric oxygen in the presence of phosphate, but found glucose to be unaffected. In view of these experiments Meyerhof and Matsuoka [1924] again brought forward the hypothesis first proposed by Neuberg [1924] that glucose is converted to fructose before undergoing oxidation *in vivo*. Glucose in the presence of glycine undergoes caramelisation at 37°, even at reactions less alkaline than p_H 8, which suggests that significant changes are effected by glycine in the reactivity of the glucose molecule, of which possibly one may be an increased susceptibility to oxidation. This increased oxidisability certainly exists in the amino-acids. The yield of ammonia from the oxidation of glycine by hydrogen peroxide and ferrous sulphate was increased from 8 % of the total possible when it was oxidised alone, to 25 % when the oxidation was carried out in the presence of glucose and phosphate. Neither urea nor cyanic acid was found even when the oxidation resulted in 25 % deamination.

EXPERIMENTAL.

The first observations on the effect of glucose in reducing the free amino-N of protein derivatives were made during a repetition, in modified form, of the experiments of Kostytcheff and Brilliant [1914] on the influence of glucose on the enzymic synthesis of protein. The following is a typical result.

Table I. *Effect of glucose in reducing free amino-N.*

Exp.	p_H	Concentrated peptic digest of albumin cc.	Glucose g.	Hog liver powder g.	Free amino-N after 48 hours' incubation at 37° mg.
1	8·4	10	4	0·1	56·5
2	8·4	10	—	0·1	154·6
3	8·4 (+4·0)	10	4	0·1	114·0
4	8·4 (+4·0)	10	—	0·1	166·9
5	4·0	10	4	0·1	160·1
6	4·0	10	—	0·1	161·0

Pfanstiehl's "C.P." glucose was used. The liver powder, as will be shown later, was not a factor here. The free amino-nitrogen was estimated by the method of Van Slyke in the macro-apparatus. The p_H of Exps. 3 and 4 was changed from 8·4 to 4·0 after 24 hours' incubation. The dilution was maintained constant in all. Toluene and chloroform were employed as preservatives.

The small reduction in free amino-nitrogen in Exp. 2 was probably due to a synthetic reaction, as analysis showed some increase in the protein content.

The interaction between glucose and the amino-group takes place only in alkaline solution. This effect of the hydrogen ion concentration is shown in Table II.

Table II. *Effect of c_H on the interaction of glucose and the amino-group.*

p_H	Amino-N after incubation with glucose. % of total nitrogen
Control (p_H 7·0)	18·5
5·0	18·3
6·3	17·5
7·1	15·1
7·8	12·8
8·4	12·4

To 10 cc. of a concentrated peptic digest of albumin 4 g. Pfanstiehl "C.P." glucose were added with chloroform and toluene, and incubated at 37° for 48 hours. The liver powder, also added to these solutions, effected some small amounts of synthesis, which accounts for the slight reductions in the free amino-nitrogen in the acid solutions. The free amino-nitrogen was estimated by formaldehyde titration.

In Table III the results of fractional analysis of a peptic digest after incubation with glucose show quite definitely that there are no indications of a synthetic reaction of sufficient extent to account for the large reduction in free amino-nitrogen.

Table III. *Fractional analysis of peptic digest after incubation with glucose.*

Exp.	Concentrated peptic digest of albumin p_H 8·5 cc.	Glucose g.	Hog liver powder g.	Free amino-N of mixture	Protein-N	Proteose-N	Peptone-N	Sub-peptone-N
				Percentage of total nitrogen				
1	30	12	0·6	9·6	6·4	42·2	27·9	24·0
2	30	12	—	9·8	5·4	43·8	29·9	20·8
3	30	—	0·6	17·2	3·8	52·0	23·9	21·3
4	30	—	—	17·4	3·6	53·1	23·9	19·4

Pfanstiehl glucose was used. The differences between Exps. 1 and 2, and 3 and 4, in the values for the proteose and peptone fractions, are due to the increased solubility of proteose in the presence of glucose, which prevents a maximum salting out. This increased solubility has been observed also with albumin, Witte's peptone and Parke-Davis' peptone, and is probably the result of the affinity of the glucose for the amino-group. No increase in ammonia was found (the titrations were carried out with $N/14$ acid and alkali) in any of the experiments, nor was any urea or cyanic acid detected.

A comparison of the free amino-nitrogen values in Exps. 1 and 2 in Table III shows that the decrease in free amino-N is as great in the absence of enzyme as when it is present. This is again demonstrated in Table IV. The peptic digest of albumin was boiled for half an hour. Two specimens of glucose were used, one, Baker's "C.P.," the other prepared from technical glucose by two recrystallisations according to the method of Hudson and Dale [1917].

Table IV. *Action of glucose in the absence of enzyme.*

Exp.	Glucose used	Free amino-N in 10 cc. of digest after incubation mg.	p_H Before incubation	p_H After incubation
1	—	66·6	8·3	8·3
2	"C.P."	37·1	8·3	7·2
3	Recrystallised	37·1	8·3	7·2

In Exps. 2 and 3, 10 cc. of the digest and 4 g. of sugar were used. No. 1 was without sugar. The mixtures were incubated for 48 hours at 37°. The fall in p_H in Exps. 2 and 3 is to be expected from the removal of the amino groups.

The dependence of this interaction of glucose and amino-nitrogen on excess of hydroxyl ions, shown in Table II, is found again when glycine is substituted for a peptic digest as the carrier of the amino-group. In Table V are given the results after incubation of a number of 1 % glycine solutions in $M/2$ phosphate at various hydrogen ion concentrations with 13·2 % glucose (Baker's "C.P."). Chloroform was added to these mixtures; the flasks were tightly stoppered and incubated at 37°: electrometric p_H determinations and free amino-nitrogen estimations (Van Slyke's method) were made from time to time. As a control, a parallel series of glycine solutions at the same hydrogen ion concentrations containing identical amounts of phosphate, but without sugar, was observed. The free amino-nitrogen values given are corrected for the change in volume due to the added glucose.

Table V. *Effect of c_H on the interaction of glucose and glycine.*

Time hours	p_H	Free amino-N in 10 cc.	
		With glucose mg.	Without glucose mg.
0	7·8	9·4	9·5
20	7·8	8·5	9·5
46	7·8	8·1	9·4
0	7·9	9·4	9·4
22	7·9	8·4	9·4
46	7·9	7·7	9·4
0	8·3	9·4	9·4
16	8·3	8·1	9·4
48	8·3	7·5	9·4
0	8·7	9·5	9·5
19	8·7	7·7	9·4
48	8·7	7·4	9·4
0	9·2	9·0	9·2
19	9·2	5·8	9·2
46	9·2	5·6	9·2

To carry the p_H range further, another series of 1 % glycine solutions in $M/2$ phosphate with and without sugar was prepared, and incubated for 48 hours at 37°. The p_H values given in Table VI are those of the phosphate solutions before the addition of glucose or glycine. The free amino-nitrogen values, as in Table V, are corrected for the volume changes due to dissolved sugar.

Table VI. *Effect of c_H on the interaction of glucose and glycine.*

p_H	Free amino-N (in 10 cc. of 1 % glycine after incubation at 37° for 48 hours)	
	With glucose mg.	Without glucose mg.
9·2	6·7	9·6
10·0	6·0	9·5
10·6	3·9	9·5
11·0	2·5	9·3

After incubation with glucose in alkaline solution and suffering reduction in free amino-nitrogen, glycine, peptic digest, peptone and albumin acquire the property of reducing methylene blue without the aid of an enzyme. In these experiments the preliminary incubation and the subsequent treatment with methylene blue were performed with an aseptic technique, controlled by tests for organisms and spores. The reagents were heated for half an hour in a boiling water-bath, and chloroform and toluene were added to the final mixtures. In Table VII are stated the details of the mixtures and the results.

The peptic digest, free from material precipitable by trichloroacetic acid, was prepared from a 3 weeks' peptic digest of 3 % albumin. It was neutralised to litmus and concentrated over a boiling water-bath to one-tenth of the original volume. The glycine solution contained 14·4 g. Pfanstiehl C.P. glycine in 100 cc. of $M/2$ K_2HPO_4. The concentrated digest and the glycine solutions were adjusted to p_H 8·5. The glucose employed was Baker's C.P.

Table VII. *Reduction of methylene blue by incubated mixtures of glucose and glycine or peptone.*

						Free amino-N		Time to decolorise 0·05% methylene blue	
Exp.	Concentrated peptic digest of albumin cc.	14·4 % glycine cc.	$M/2$ K_2HPO_4 cc.	Glucose g.	p_H after incubation and dilution	before heating in acid solution mg.	after heating in acid solution mg.	before heating in acid solution	after heating in acid solution
1	10	—	—	4	8·0	25	63	48 hrs.	Partial in 24 hrs.
2	10	—	—	4	8·0	30	62	48 hrs.	Partial in 24 hrs.
3	—	—	10	4	9·7	—	—	No decolorisation	No decolorisation
4	—	10	—	4	7·5	193	198	15 mins.	19 mins.
5	—	10	—	4	7·5	192	198	15 mins.	19 mins.
6	—	10	—	—	8·4	254	251	No decolorisation	No decolorisation
7	10	—	—	—	8·4	45	62	No decolorisation	No decolorisation
8	10	—	—	4	—	—	—	Partial in 48 hrs.	—
9	—	2·5	—	1	—	—	—	24 hrs.	—

After 48 hours' incubation in a water-bath at 37° sterility tests were made on 1 cc. removed aseptically from each of the above solutions, and the remainder was diluted to 100 cc. with $N/280$ NaOH.

Solutions nos. 8 and 9 were prepared at this time. In solution no. 8, 4 g. sugar were dissolved in 10 cc. $N/280$ NaOH and 10 cc. peptic digest, and the resulting solution diluted to 100 cc. with $N/280$ NaOH. Solution no. 9 consisted of 1 g. glucose and 2·5 cc. of the control 14·4 % glycine solution (no. 6 in Table VII) diluted to 25 cc. with $N/280$ NaOH.

For the determination of the time required to decolorise methylene blue, 10 cc. of the solutions described above and in Table VII were pipetted into 10 cc. of 0·05 % methylene blue, in 25 cc. test tubes. These were tightly stoppered with rubber corks and placed in a water-bath at 56°. To 40 cc. (except nos. 8 and 9) 1 cc. of conc. HCl was added and the mixtures heated for 1 hour in a boiling water-bath and then left overnight in an ice-chest. The added acid was then neutralised, and the resulting solution made up to 50 cc. with distilled water. Methylene blue was added to 10 cc. of these as in the unboiled solutions. The free amino-nitrogen of both boiled and unboiled mixtures was estimated by the Van Slyke method, and the results are expressed in terms of 10 cc. of original undiluted peptic digest and glycine respectively. The p_H values given in Table VIII are those of the incubated solutions after dilution with $N/280$ NaOH.

At the end of the 48 hours' incubation all solutions were found to be free from organisms or spores. Solutions 4 and 5 had become dark reddish brown and possessed the odour characteristic of solutions containing caramel. The results of the treatment of solutions 4 and 5 show also that the compound, which arises during incubation of glucose with glycine, and which reduces methylene blue, is not destroyed by heating with acid. The result with solution 9 compared with 4 and 5 shows that the preliminary 48 hour incubation reduced the time required to decolorise methylene blue from 24 hours to 15 minutes.

In the case of the peptic digest, experiments 1 and 2, the reduction of methylene blue was more sluggish, and the amino-nitrogen lost during incubation was restored by heating in acid solution. These differences between peptic digest and glycine are in large part due to the absence of phosphate from the peptic digest mixtures in 1 and 2, as is clearly shown in Table VIII.

Table VIII. *Effect of phosphate on the reduction of methylene blue by peptone and glucose.*

					Free amino-N			Time to decolorise 0·05 % methylene blue	
Exp.	Digest	Glucose g.	$M/2$ K_2HPO_4 p_H 8·5 cc.	14·4 % Glycine cc.	before heating with acid mg.	after heating with acid mg.	p_H	before heating with acid	after heating with acid
1	With phosphate	—	—	—	73·5	85·6	8·2	No decolorisation	No decolorisation
2	,,	8	—	—	24·6	62·8	7·5	22 mins.	23 mins.
3	,,	8	—	—	24·4	64·9	7·5	18 mins.	19 mins.
4	Without phosphate	8	—	—	44·8	107·2	7·4	Partial in 24 hrs.	None in 24 hrs.
5	,,	8	—	—	44·4	105·3	7·4	Partial in 24 hrs.	None in 24 hrs.
6	,,	—	—	—	73·5	108·8	8·5	No decolorisation	No decolorisation
7	—	8	8	—	—	—	9·1	Slight in 24 hrs.	Slight in 24 hrs.
8	—	2	2	5	207·8	—	7·4	18 mins.	—

Two 30 % solutions of Witte's peptone were made up, one with $N/14$ NaOH, the other with 0·75 M K_2HPO_4. The reaction in both was adjusted to p_H 8·5. The solutions described in Table VIII were made with the same aseptic technique as those in Table VII, and incubated, as there, for 48 hours in a water-bath at 37°. Solutions 1, 2 and 3 contain, however, more total N than the corresponding ones in Table VII, because no withdrawal of solution was made for sterility tests. Solutions 2 and 3 were much less turbid than 1 on account of the increased solubility of peptone in the presence of glucose. Solution 8 served as a control for the activity of the whole system.

After 48 hours all except solution 8 were diluted with $N/280$ NaOH to 100 cc. and number 8 was diluted to 50 cc. The free amino-nitrogen estimation, the methylene blue reduction, the p_H determinations, and the heating in acid and subsequent neutralisation and dilution were carried out in exactly the same manner as in the experiments described in Table VII.

In the presence of phosphate the reduction of methylene blue by peptone and glucose is as rapid as the reduction by glycine, glucose and phosphate. Less of the amino-nitrogen lost by the peptone during incubation is restored by boiling with acid when phosphate is present. The increased value for free amino-nitrogen after boiling with acid in solutions 4, 5 and 6, as compared with solutions 2 and 3, is probably accounted for by the increased hydrolysis in the greater acidity due to the absence of buffer.

With protein, phosphate is not as essential for the rapid reduction of methylene blue after incubation with sugar. The reduction in free amino-nitrogen in the preliminary incubation with glucose, if any, is quite small.

Two suspensions of egg albumin were prepared, of approximately 30 % concentration, one in $M/4$ K_2HPO_4, the other in dilute NaOH, both with a final p_H of 8·5. The protein was more soluble in the phosphate than in the NaOH, and both protein solutions were considerably clearer after addition of glucose.

A number of solutions were made, and incubated in a water-bath at 37° for 48 hours. The details of the solutions and the results are noted in Table IX.

Table IX. *Reduction of methylene blue by albumin and glucose.*

					Free amino-N			Time to decolorise 0·1 % methylene blue	
Exp.	Albumin with K_2HPO_4 cc.	Albumin without K_2HPO_4 cc.	Glucose g.	K_2HPO_4 p_H 8·5 cc.	before heating with acid mg.	after heating with acid mg.	p_H	before heating with acid	after heating with acid
1	20	—	8	—	1·4	1·7	8·1	55 mins.	—
2	—	20	8	—	1·5	1·2	9·1	38 mins.	40 mins.
3	20	—	—	—	1·8	1·7	9·2	Partial. 0·01 % in 24 hrs.	No decolorisation
4	—	20	—	—	1·5	1·9	10·0	Partial. 0·01 % in 24 hrs.	No decolorisation
5	—	—	8	20	—	—	9·1	0·01 % in 46 mins.	0·01 % in 46 mins.

The treatment after incubation was the same as in the previous experiments.

The free amino-nitrogen values are uncertain. The faster reduction of methylene blue in the solution containing phosphate was due more probably to the higher alkalinity than to the phosphate.

An attempt was made to ascertain whether a similar power to reduce methylene blue was possessed by acetamide, urea and creatine after incubation with glucose. No attempts were made to measure any changes that might have occurred in the NH_2 group. It was found that acetamide, urea and creatine after this treatment, did not reduce methylene blue, even in the presence of phosphate, at a rate appreciably faster than glucose alone, or at a less alkaline p_H.

Table X. *Effect of washed muscle on reduction of methylene blue by peptone and sugar.*

Exp.	Digest cc.	Inc. sugar	Sugar g.	$M/2$ K_2HPO_4 p_H 8·5 cc.	Washed muscle (enzyme) g.	Time to decolorise 0·01 % methylene blue
1	10	+	—	—	2	3½ hrs.
2	10	+	—	—	—	10 hrs.
3	10	—	+	—	2	Nearly complete in 24 hrs.
4	10	—	+	—	—	Partially in 24 hrs.
5	—	—	1	10	2	No decolorisation
6	10	—	—	—	2	No decolorisation
7	—	—	1	10	2	Slight decolorisation in 24 hrs
8	—	—	—	10	2	No decolorisation

The decolorisation of methylene blue by peptone after incubation with glucose is accelerated by the addition of washed muscle prepared by the method of Thunberg [1917]. A concentrated peptic digest of albumin was incubated at an initial p_H of 8·3 with 10 % glucose for 24 hours at 37°. The solution was then diluted with an equal volume of $M/2$ K_2HPO_4 and heated at 56° for 1 hour to drive off the chloroform and to sterilise the mixtures. At the same time 50 cc. of digest without sugar were similarly treated and, after heating, glucose to a concentration of 10 % added. The p_H of both solutions was finally 7·9. In Table X "inc. sugar" designates digest incubated with glucose for 24 hours, and "sugar" the digest to which glucose was added immediately before the addition of the washed muscle.

Preliminary incubation with glucose increases the yield of ammonia after treatment with H_2O_2 and ferrous sulphate. A typical result is given in Table XI.

Table XI. *Effect of glucose on the oxidation of glycine by* H_2O_2.

Exp.	1 % glycine in K_2HPO_4 cc.	Glucose g.	30 % H_2O_2 cc.	H_2O cc.	Ammonia-N mg.
1	25	3·1	8	2	5·8
2	25	2·0	6	4	5·7
3	25	1·25	4	6	2·2
4	25	0·75	2	8	2·9
5	25	0·25	2	8	2·5
6	25	—	2	8	2·2
7	25	3·1	—	10	0·4

A 1 % glycine solution in $M/2$ K_2HPO_4 at p_H 8·8 was used. All the solutions were incubated for 24 hours at 37°. At the end of this period there still remained some undecomposed H_2O_2 which was removed by freshly precipitated manganese dioxide. The ammonia was determined by aeration in a strongly alkaline solution, and the urea by precipitation with xanthhydrol. The cyanic acid was estimated by incubation with excess of ammonium chloride and precipitation of the resulting urea with xanthhydrol. The ammonia formed in Exps. 1 and 2 corresponds to 17·5 % of the total nitrogen. No cyanic acid or urea was found.

The dependence of the oxidation of glycine and glucose by H_2O_2 on the reaction is shown in Table XII.

Table XII. *Effect of* p_H *on the oxidation of glycine and glucose by* H_2O_2.

Exp.	p_H	Ammonia-N formed; percentage of total N
1	9·5	19·1
2	8·7	17·6
3	8·0	17·2
4	7·7	14·1
5	7·2	9·5
6	6·9	7·6
7	6·3	4·4
8	5·9	5·7
9	3·8	2·4

1 % glycine solutions were made up in $M/2$ K_2HPO_4 at various p_H values and the final p_H in each case determined electrometrically. To 30 cc. of each of these solutions 3·75 g. glucose (Pfanstiehl C.P.) and 10 cc. of neutralised 30 % H_2O_2 and one crystal of ferrous sulphate were added. After 24 hours' incubation at 37°, the solutions were neutralised and the excess H_2O_2 was

decomposed with freshly precipitated manganese dioxide. The ammonia was determined by aeration and the urea and cyanic acid by xanthhydrol. Neither urea nor cyanic acid was found.

SUMMARY.

1. In alkaline solution, in the absence of any enzyme, an interaction occurs between glucose and protein derivatives which results in a reduction of the free amino-nitrogen. The reduction is not the result of a synthetic process and neither urea, cyanic acid nor ammonia is formed.

2. Simultaneously with the reduction in free amino-nitrogen the mixture of glucose and protein derivative acquires the property of reducing methylene blue in approximately neutral solution.

3. With glycine and peptone this reduction of methylene blue is accelerated by phosphate. With albumin an equal velocity of reduction is obtained without phosphate.

4. The reduction of methylene blue by incubated peptone and glucose is accelerated by washed muscle.

5. Preliminary incubation with glucose increases the yield of ammonia after oxidation with hydrogen peroxide.

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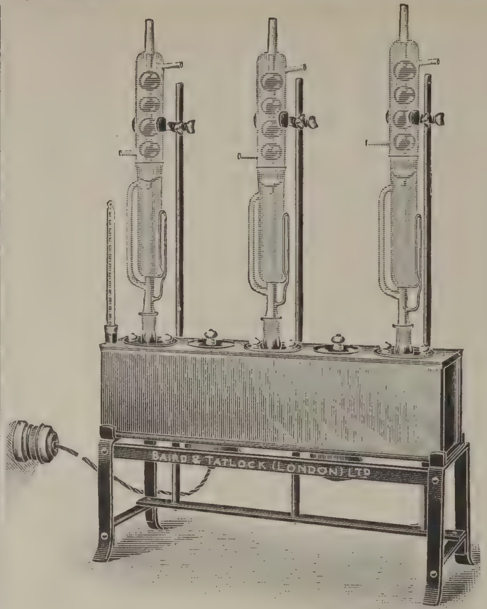

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